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Th17 immune responses in the chicken

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A dissertation submitted for the degree of Doctor of Philosophy

University of Edinburgh

2015



Author's declaration

I declare that the work in this dissertation is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree.

Signed.....

Date.....

Abstract

In recent years, the subsets of mammalian CD4⁺ T cells and their repertoire of effector cytokines has expanded beyond the original Th1/Th2 paradigm, to include natural (n) and inducible (i) regulatory T cells (Treg), Th17, Th9, Th22 and follicular T helper (Tfh) cells. Whilst Th1, Th2 and nTreg immune responses have been described in the chicken, the existence of other Th cell subsets is yet to be determined. To investigate Th17 immune responses in the chicken, the mammalian components of these responses currently unannotated in the chicken genome, IL-23 p19 and the IL-23R, were identified and cDNAs cloned. A chicken IL-23 flexi-construct, containing IL-23 p19 and p40 joined by a linker, was designed. Recombinant chicken IL-23 protein (rchIL-23) was expressed and purified. Bioactivity of rchIL-23 was demonstrated by increased mRNA expression of chIL-17F and chIL-22 in rchIL-23-stimulated splenocytes. Monoclonal antibodies which identify chIL-12/chIL-23 p40 also recognised purified rchIL-23. Further, chIL-23 p19 mRNA levels were measured and detected in a wide range of tissues but was not up-regulated in stimulated splenocytes, thymocytes or bursal cells.

Messenger RNA (mRNA) expression levels of Th17 cytokines (chIL-17A, chIL-17F, chIL-21, chIL-22 and chIL-23) were measured in a chicken tissue panel, in stimulated splenocytes, thymocytes and bursal cells, as well as during infections previously described as initiating typical Th1 or Th2 adaptive immune responses in the chicken. Chicken IL-17A mRNA expression levels were up-regulated in susceptible chickens during infection with Marek's disease virus (a disease which typically drives a Th1 immune response), but were down-regulated in resistant birds.

Chicken CD4⁺ T cells were sorted by fluorescence-activated cell sorting (FACS) and recombinant Th17-associated cytokines used to attempt to drive the cells towards a Th17 phenotype, as measured by expression of mRNA for chIL-17A and chIL-23R. The sorted chicken CD4⁺ cells failed to proliferate or respond to Th17 cytokine stimulation.

ChIL-23R was also correctly identified and cloned as cDNA, and its mRNA expression measured in a panel of unstimulated and stimulated tissues and cells. The chIL-23R mRNA levels were detected in a wide range of tissues as well as stimulated splenocytes, thymocytes and bursal cells. Future work would seek to positively identify Th17 cells in the chicken and determine the role of Th17 immune responses against avian diseases.

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Acknowledgements

The first and most important thank you must go to my two supervisors Professor Pete Kaiser and Lisa Rothwell for their support and guidance throughout my PhD. I am also extremely grateful to Professor John Hopkins, my third supervisor, who stepped into the breach in the final throes of writing without a word of complaint and always a smile.

A special thank you to Dr Lonneke Vervelde for the metaphorical hand holding, and for challenging me to think scientifically in the last 12 months of lab work. A mention must also go to the Animal Infectious Diseases Group for making me laugh in the face of scientific adversity many times. I would also like to acknowledge Dr Darren Shaw for the calm approach to the last minute help with statistical analyses, which had literally reduced me to tears.

I am very lucky to have some loyal and unwavering friends who have gone above and beyond to help me during my PhD. Without your love and faith in me, this PhD would not have been possible. A special mention to those few who have been by my side daily: Maggie, for never a quiet day; Kate, for the excellent craic; Robin, for the daunting task of proof reading my thesis; Anna, for helping me negotiate the world of FACS and Lottie, for always being there with a sensible word.

Last, and by no means least, I would like to thank my family. Dad, Mum, Mike and Clare, I could not have come this far without your support. It has been an educational journey lasting over 10 years, I promise to finally leave university now.

Abbreviations

α	Alpha
aa	Amino acid
Ab	Antibody
Ag	Antigen
Ahr	Aryl hydrocarbon receptor
amp ^r	Ampicillin resistance gene
APC	Antigen presenting cells
ATP	Adenosine triphosphate
β	Beta
BAC	Bacterial artificial chromosome
BALT	Bronchial associated lymphoid tissue
BCO	Bacterial chondronecrosis with osteomyelitis
BCR	B cell receptor
BLAST	Basic local alignment search tool
BMDCs	Bone marrow-derived dendritic cells
bo	Bovine
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CDS	Coding sequence
CEF	Chicken embryonic fibroblasts
ch	Chicken
CLR	C-type lectin receptor
CD	Cluster of differentiation
CFU	Colony forming units
cm	Centimetre
CM	Conditioned medium
CMV	Cytomegalovirus
CO ₂	Carbon dioxide
ConA	Concanavalin A
CS	Chicken Serum
CSF	Colony-stimulating factor
C _t	Cycle threshold
CTL	Cytotoxic T lymphocytes
δ	Delta
d	Day
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dpi	Days post-infection
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis

ECL	Enhanced chemiluminescence
<i>E. coli</i>	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced Green Fluorescent Protein
EID	Embryonic incubation day
ELISA	Enzyme-linked immunosorbent assay
EST	Expressed sequence tag
ex-COS	Derived from COS-7 cells
FACS	Fluorescence-activated cell sorting
FAM	5- or 6-carboxyfluorescein
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
FSC	Forward scatter
γ	Gamma
g	Gram
<i>g</i>	Relative centrifugal force
GATA3	trans-acting T-cell-specific transcription factor GATA-3
G-CSF	Granulocyte -colony stimulating factor
GALT	Gut-associated lymphoid tissues
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	Hours
<i>H. meleagridis</i>	Histomonas meleagridis
HEK	Human embryonic kidney
HRP	Horseradish peroxidase
hu	Human
H ₂ O	Water
IBD	Inflammatory bowel disease
IBDV	Infectious bursal disease virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
IPTG	Isopropyl-beta-D-1-thiogalactopyranoside
IRAK1	IL-1 receptor-associated kinase
iTreg	Inducible Treg cells
JAK	Janus kinase
l	Litre
LB	Lysogeny broth
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
M	Molar
mAbs	Monoclonal antibodies
MACS	Magnetic-activated cell sorting
MAD	Mothers against decapentaplegic protein
MAPK	Mitogen-activated protein kinase
MCS	Multiple cloning site

MD	Marek's disease
MDV	Marek's disease virus
mg	Milligram
MgCl ₂	Magnesium chloride
MHC	Major histocompatibility complex
min	Minute
ml	Millilitre
m	Mouse
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NET	Neutrophil extracellular trap
ng	Nanogram
NK	Natural killer cells
NKT	Natural killer T cells
NLR	NOD-like receptor
nm	Nanometre
NOD	Nucleotide oligomerisation and binding domain
NTC	No template control
nt	Nucleotide
nTreg	natural Treg cells
°C	Degree (s) Celsius
OD	Optical density
ORF	Open reading frame
p	Pico
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline and Tween20
PCR	Polymerase chain reaction
PE	R-Phycoerythrin
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
poly(A)	Polyadenylation
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
RT-qPCR	quantitative real-time RT-PCR
r	Recombinant
R	Receptor
RACE	Rapid amplification of cDNA ends
RAG	Recombination activating gene
RAR	Retinoic-acid-related
rch	Recombinant chicken protein
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like helicase receptor
RNA	Ribonucleic acid
ROR γ t	RAR-related orphan receptor gamma t
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute

rRNA	ribosomal ribonucleic acid
RT	Reverse transcriptase
s	Second
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SOC	Super-optimal broth with catabolite repression
SMA	<i>Caenorhabditis elegans</i> SMA protein
SNP	Single nucleotide polymorphism
SPF	Specific pathogen free
STAT	Signal transducer and activator of transcription
SV40	Simian virus 40
TAE	Tris-acetate-EDTA
TAMRA	Tetramethylrhodamine
<i>Taq</i>	<i>Thermus aquaticus</i>
TBE	Tris-borate-EDTA
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cell
TIR	Toll/IL-1 receptor homology
TLR	Toll-like receptor
Tm	Melting temperature
TNF	Tumour necrosis factor
TR1	Type 1 regulatory T cells
Treg	Regulatory T cell
Tyk	Tyrosine kinase
UTR	Untranslated region
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
μ	Micro

Chapter 1 Introduction

Globally, chickens are one of the most important sources of protein, in the form of both meat and eggs, and are consequently a major resource of food. Around 52 billion chickens are reared per year and this immense supply and demand for poultry is increasing; approximately 91 million tonnes of chicken meat were consumed across the globe in 2012. In the UK alone there are over 850 million chickens produced for meat each year (Kaiser, 2010) and approximately 29 million laying hens generate over 1.1 billion eggs. As a result, poultry health and welfare are fundamental to the supply and safety of food.

Poultry farming is carried out on an industrial scale and birds are commonly farmed under very intensive conditions. In today's world, it takes only 40 days for a broiler chicken to reach its target weight of 2 kg for slaughter. Layers have a longer lifespan and typically produce around 300 eggs per year. This level of poultry production is due to constant genetic selection for growth rates by chicken breeders, better understanding and quality of poultry nutrition and improved disease control programmes. Intensive rearing and processing techniques have also contributed to the massive increase in poultry production. With so many birds in close proximity to each other and under such growth pressure, the potential for disease outbreak is enormous. The financial implication of avian-specific disease in the poultry industry is great, despite routine vaccination against many different pathogens. More important is the zoonotic transmission of disease to humans via the food-chain. Zoonotic diseases such as salmonellosis and campylobacteriosis are well-recognised causes of food poisoning, mainly as a result of contaminated poultry products.

Understanding the pathogenesis of avian disease and the immunobiology of the chicken will improve food security.

The nature of the infectious challenge to the poultry industry is constantly changing due to environmental change, changes in legislation and pathogen evolution. For example, legislation introduced nearly 10 years ago in the UK banned the use of antimicrobials as growth promoters in food-producing animals. Whilst effective at slowing evolution of antimicrobial drug resistance in humans, this increases the risk of disease in food-producing animals and consequently of disease transmission via the food chain to humans. With the increasing demand on poultry production, strategies to preserve food security are vital. One of the keys to developing effective disease control measures for the future is a better understanding of the avian immune system and how to manipulate it.

1.1 The mammalian immune system

All living organisms have evolved mechanisms and strategies to defend themselves against disease. In concert, pathogens are continually evolving their own survival strategies to evade these immune defences. Healthy co-evolution is reached in some circumstances, e.g. commensal bacteria, but more commonly there is a continual “arms-race” between host and pathogen. The immune system is classically divided into two sub-systems: the innate immune response and the adaptive immune response.

1.1.1 *The innate immune response*

The innate immune system is the first cellular line of defence against invading pathogens. It is an immediate, non-antigen-specific system. The innate immune response does not confer immunological memory, nor does it alter on repeated exposure to the same pathogen. It is an evolutionarily older defence mechanism than the adaptive immune system (section 1.1.2).

The innate immune cells which orchestrate pathogen recognition and the host's response include epithelial cells, mast cells, macrophages, dendritic cells (DCs), neutrophils, basophils, eosinophils, gamma delta ($\gamma\delta$) T cells and Natural Killer (NK) cells. The phagocytes (macrophages, DCs and neutrophils) identify, engulf and eliminate pathogens as well as presenting antigen to B and T cells to prime the adaptive immune response. Innate immune cells play key roles in effecting innate immune responses. Macrophages, DCs and epithelial cells "sense" pathogens by expression of pattern recognition receptors (PRRs) (discussed in more detail below). They also express other receptors such as complement, mannose, glucan and scavenger receptors, which recognise many microbial ligands. Upon antigen stimulation, these cells secrete a range of inflammatory mediators, e.g. cytokines, to recruit other effector cells such as neutrophils. Neutrophils are potent killers of pathogens through the release of antimicrobial molecules and neutrophil extracellular traps (NETs), which reduce the spread of infection and inflammation by killing bacteria and neutralising bacterial virulence factors (Brinkmann *et al.*, 2004). NK and $\gamma\delta$ T cells are cytotoxic lymphocytes, which functionally sit at the interface between the innate and adaptive immune responses. They are capable of driving immune responses based on the expression of non-antigen-specific pleiotropic

receptors but their functions are only just being explored. $\gamma\delta$ T cells can express high levels of cytokines and chemokines, such as interferon (IFN)- γ , tumour necrosis factor (TNF), interleukin (IL)-17 and CXCL13, as well as granzymes to lyse infected or stressed host cells. They also play roles in DC maturation, priming of $\alpha\beta$ T cells via antigen presentation and helping B cells (Vantourout and Hayday, 2013). NK cells also do not directly attack invading microbes; they destroy pathogen-infected host cells with reduced major histocompatibility (MHC) class I expression to release pathogen components, facilitating clearance by phagocytes (Vivier, 2006). NK cells have recently been assigned as prototypical Group 1 innate lymphoid cell (ILC1) (Spits *et al.*, 2013). ILC1s are developmentally related cells with important effector roles in innate immunity and tissue remodelling. They are defined by three main features: lack of recombination activating gene (RAG)-dependent rearranged antigen receptors, absence of myeloid cell and DC phenotypical markers and a lymphoid morphology (Spits and Cupedo, 2012).

To recognise and respond to pathogens, innate cells must first distinguish between self and non-self. To this end, the cells use a limited number of soluble and cell-associated receptors and proteins, encoded in the germline, which recognise common features of pathogens. Pathogens express several signature molecules, which are known as pathogen-associated molecular patterns (PAMPs). These tend to be indispensable components of microbes, which consequently are not readily altered by mutation or selection (Beutler, 2004). As mentioned previously, the receptors recognising invading pathogens, or PAMPs, are PRRs, which can also detect damage-associated molecular patterns (DAMPs). DAMPs are molecules produced by pathogen invasion or cell damage, such as reactive oxygen species (ROS) or

adenosine triphosphate (ATP) (Gombault *et al.*, 2012). To date, there are four major classes of PRRs: toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene I (RIG-I)-like helicase receptors (RLRs) and nucleotide oligomerisation and binding domain (NOD)-like receptors (NLRs) (Hardison and Brown, 2012; Lupfer and Kanneganti, 2013). Upon activation, PRRs signal to the host the presence of infection and trigger pro-inflammatory and antimicrobial responses by activating a multitude of intracellular signalling pathways, adaptor molecules, kinases and transcription factors (Akira and Takeda, 2004). Therefore, PRR activation represents an important link between the innate and adaptive immune responses.

TLRs are the most well-characterised of the PRRs and they detect a wide range of microbial structures on the exterior of bacteria, viruses, protozoa and fungi (Kawai and Akira, 2010). They are expressed by many different cell types and structurally are glycoproteins characterised by an extracellular domain containing leucine-rich repeat (LRR) motifs and a cytoplasmic signalling Toll/IL-1 receptor homology (TIR) domain (O'Neill and Bowie, 2007). One way of grouping TLRs is on their cellular distribution. TLR1, 2, 4, 5, 6 and 10 are expressed on the cell surface whereas TLR3, 7, 8 and 9 are located intracellularly (Iwasaki and Medzhitov, 2004). The TLRs expressed on the cell surface recognise products unique to bacteria, e.g. lipopolysaccharide (LPS) and flagellin, whereas the intracellular TLRs recognise pathogen-derived nucleic acids and discriminate self from non-self by the localisation of their ligands rather than a unique molecular structure different from the host (Mogensen, 2009).

In contrast, CLRs are essential for antifungal immunity. They are a heterogeneous superfamily of soluble and transmembrane proteins defined by a characteristic C-type lectin domain (Zelensky and Gready, 2005) and recognise the major carbohydrate structures found in fungal cell walls.

The RLRs sense viruses. These receptors are widely expressed cytosolic RNA helicase proteins, which lack a transmembrane domain and can identify viral ribonucleic acid (RNA). RLR activation leads to expression of anti-viral effector molecules such as type I IFN (Creagh and O'Neill, 2006; Ramos and Gale, 2011).

Similar to RLRs, NLRs lack a transmembrane domain and are cytoplasmic sensors of intracellular pathogen invasion, sensing PAMPs and DAMPs. NLRs are a large receptor family with over 20 members, which have a wide range of effector and activation domains and consequently activate multiple signalling pathways (Lupfer and Kanneganti, 2013). One of the most studied and well-known roles of NLRs is in inflammasome activation. The inflammasome is a multimeric protein complex. These complexes are able to use an activated NLR to further activate pro-caspase-1, ultimately resulting in the generation of mature IL-1 β and IL-18 pro-inflammatory cytokines (Latz *et al.*, 2013).

Following detection of invading pathogens, the innate immune response orchestrates neutralisation and removal of the pathogen as well as initiating the adaptive immune response should it fail to overcome the initial invasion. The same cells that detect foreign antigen, e.g. macrophages and neutrophils, also function to kill invading pathogens through several mechanisms touched on already such as phagocytosis, NETs and the release of effector molecules, e.g. cytokines, to activate other cells,

contributing to the developing immune response. Pathogen-activated innate immune cells produce cytokines to organise and drive the immune response to specific antigens. Cytokines are chemical messengers that include chemokines, IFNs, ILs and TNFs. Cytokines released by activated macrophages include the pro-inflammatory cytokines IL-1 β , IL-6, IL-12 and TNF- α , which initiate early inflammatory responses, the chemokine CXCL8, which attracts neutrophils to sites of infection and the interferons IFN- α and IFN- β , which block viral replication.

An additional effector component of innate immunity is the complement system. Plasma proteins, constitutively expressed by the liver, react with one another to opsonize pathogens, which induces several inflammatory responses designed to aid innate cells in their removal. Three pathways of complement activation are defined differing in how they are triggered and in the first reactions in the cascade: the classical pathway, the lectin pathway and the alternative pathway (Zipfel and Skerka, 2009). However, they all lead to the activation of the protein C3, deposition of C3b on the surface of the pathogen and the recruitment of effector mechanisms for pathogen destruction.

The outcomes of the innate immune response and its effect on influencing the nature of the adaptive immune response depends on the initial signals sensed, e.g. activation of the PRR families, phagocytosis, pro-inflammatory cytokine secretion and complement activation (Mogensen, 2009). The innate immune response is activated immediately upon encountering a pathogen and continues for approximately 96 hours, at which point infection is either effectively overcome or the adaptive immune response is triggered.

1.1.2 *The adaptive immune response*

The adaptive immune response is recruited to combat invading pathogens able to evade or overcome the innate immune response and its activities are very antigen-specific and modifiable. Further, a key feature of the adaptive immune response is the development of immunological memory, allowing a rapid and efficient response to subsequent encounters with a particular antigen. This secondary response is often stronger than the primary response to infection. It consists of antibody (Ab) responses mediated by B cells, also called humoral immunity, and cell-mediated responses predominantly orchestrated by T cells. Most effector B and T cells die when the host recovers from infection but a few cells persist as memory cells to provide long-term immunity. This ensures a rapid and protective immune response can be mounted if there is repeat exposure to the same antigen. Memory cells are more sensitive to infection, more easily activated and more abundant than naïve cells specific for the same pathogen.

Both B and T cells have the capacity to respond to almost any antigen through their antigen-specific receptors (the B cell receptor (BCR) and the T cell receptor (TCR) respectively), with repertoires of specificity that expand and contract according to requirements. Where they differ is in the manifestation of this response. B cells produce antigen-specific immunoglobulin (Ig), while T cells primarily express cytokines to drive other cells (both immune and non-immune) in a developing immune response. Genetic rearrangement of both the BCR and TCR is very similar and drives huge diversity in the system, enabling recognition of almost any antigen. Another common feature shared by B and T lymphocyte development is for

apoptosis to be the default pathway unless a positive signal for survival and further differentiation is received.

B cell development in humans begins in the bone marrow. Pluripotent haematopoietic stem cells give rise to common lymphoid progenitor cells, which have the potential to differentiate into B cells (as well as most other immune cell types). Once committed to the B cell lineage, B cell progenitors (pro-B cell) undergo rearrangement of the Ig heavy-chain and light-chain genes, which results in immature B cells expressing the membrane-bound Ig, IgM. The IgM further associates with Ig α and Ig β signalling proteins to form a functional BCR complex. Non-lymphoid stromal cells provide the specialised environment for B cell maturation in the bone marrow. B cells undergo two 'checkpoints' in the bone marrow before migration to secondary lymphoid tissues. The first ensures only cells with a functional pre-B cell receptor are able to proceed in development, while the second checkpoint is assembly of a functional BCR. B cells that fail at either of the checkpoints undergo apoptosis in the bone marrow. In addition, before migrating to peripheral lymphoid organs to finish development, immature B cells must undergo negative selection to eliminate any self-reactive cells. These transitional B cells migrate to peripheral lymphoid organs, such as the spleen, lymph nodes and Peyer's patches, under the guidance of chemokines, e.g. CCL21, expressed by stromal cells. These immature B cells are considered naïve (not yet exposed to antigen). Once inside the spleen or lymph node, B cells congregate in primary lymphoid follicles where they mature. In the absence of its specific antigen, a B cell will exit in the efferent lymph. A mature naïve B cell expresses both membrane-bound IgM and IgD. Activated B cells can express and secrete different classes of Ig. This Ig class-

switching is orchestrated by cytokines. In mammals, there are five classes of Ig: IgA, IgD, IgE, IgG and IgM. Activation of a mature B cell occurs when it recognises antigen in conjunction with signals from Th cells. This process triggers proliferation and differentiation of the activated B cell into a plasma cell, an antibody-producing cell or a memory cell, which survives for years and ‘remembers’ one specific antigen. Immunoglobulins from a single B cell have the same antigen specificity. The BCR, or Ig, is formed of a dimerised heavy and light chain, with constant (C) and variable (V) regions. The heavy and light chains are derived from somatic recombination of families of gene segments, called V and joining (J) genes, and, in the case of the heavy chain, diversity (D) genes. This V(D)J recombination produces incredible diversity in antigen recognition. Antigen recognition is further “fine-tuned” in an immune response through point mutations occurring in the V, D and J genes (somatic hypermutation) of the BCR during activated B cell expansion. The classification of the five Ab isotypes is based on the C region of the different heavy chains, which also defines specific functionality of the Ab. Secreted Ig can bind to pathogens, either neutralising or opsonising the pathogen, which aids in phagocytosis, or activates complement, ultimately clearing the pathogen from the host.

T cell development also begins with lymphocyte progenitors in the bone marrow and has commonality with B cell development. T cells must also undergo gene rearrangement to produce a TCR, a process called somatic DNA recombination, which occurs after migration to the thymus. The stage of thymocyte development can be classified based on the expression of cluster of differentiation (CD) markers on the cell surface. Immature thymocytes are double-negative cells, they do not express

CD4 or CD8 (CD4⁻CD8⁻). Similarly to B cells, T cell development depends on signals received from thymic stromal cells. Activation of Notch 1, a cell-surface receptor expressed by developing thymocytes, is crucial for T cell differentiation. Double-negative thymocytes begin developing into two functionally different lineages of T cells, which are distinguished by the expression of an $\alpha\beta$ or $\gamma\delta$ TCR. At this stage, $\gamma\delta$ T cells leave the thymus and circulate in the blood as they are not subject to the stringent selection imposed on the $\alpha\beta$ T cells. If a functional TCR is not generated, the T cell dies through apoptosis. When an immature $\alpha\beta$ -expressing thymocyte loses dependence on Notch 1, it then differentiates into a CD4⁺CD8⁺ double-positive cell. Double-positive cells with a functional $\alpha\beta$ TCR are exposed to endogenous peptides expressed on thymic cells and undergo further positive and negative selection processes. Like B cells, T cells undergo selection to quality control the recombination process. During positive selection, $\alpha\beta$ T cells that are able to recognise peptides presented by self-MHC molecules are selected for (further discussion on the MHC is included below). This TCR interaction results in loss of either the CD4 or the CD8 co-receptor molecule, generating a single-positive cell (CD4⁺CD8⁻ or CD4⁻CD8⁺). The cells then undergo negative selection, with $\alpha\beta$ CD8⁺ or CD4⁺ T cells that recognise normal self-peptides presented by MHC complexes on healthy cells being eliminated. In this way, only T cells that recognise pathogen-derived peptides presented by MHC class I or II isoforms are expressed. Following selection, surviving naïve T cells enter the circulation and migrate to peripheral lymphoid organs.

There are three groups of cells in mammals considered to be antigen presenting cells (APCs): DCs, macrophages and B cells. Because DCs are unparalleled in their ability

to interact with T cells in peripheral lymphoid organs, they are considered to be the primary APC (Lewis *et al.*, 2014). APCs are vital for bridging the innate and adaptive immune responses. They express numerous PRRs to detect and phagocytose pathogens, including TLRs (see section 1.1.1). Activated DCs take up antigen in peripheral tissues before migrating to peripheral lymphoid organs to present the processed antigen to naïve T cells. APCs present antigen via MHC molecules, which are recognised by the TCR of a naïve T cell. MHC molecules are classed as either type I or type II depending on the antigen they present. MHC class I presents antigen from intracellular pathogens and is expressed by virtually all nucleated cells in the body. MHC class II is constitutively expressed by APCs and presents antigen from extracellular pathogens.

As described before, single-positive $CD8^+$ or $CD4^+$ T cells develop in the thymus. $CD8^+$ T cells are predominantly cytotoxic T lymphocytes (CTL) and are activated via MHC class I molecules. $CD4^+$ T cells are helper Th cells, which are activated via MHC class II molecules. Ligation of the TCR with its specific antigen:MHC complex is necessary to activate a naïve T cell (signal 1) but is insufficient stimulus on its own. A co-stimulatory signal from the same APC (signal 2) is also required to initiate clonal expansion and further differentiation of a naïve T cell into an effector T cell. The co-stimulatory receptor on the cell surface of a naïve T cell is CD28, which is activated by co-stimulatory molecules called B7 molecules, which are expressed on the surface of APCs.

The effector function of activated cytotoxic T cells is to kill cells that harbour intracellular infection. This is particularly important during viral infection. Binding of the $CD8^+$ TCR with MHC class I provokes a cell-mediated response where CTLs

secrete cytotoxic granules, containing molecules such as perforin, to directly kill infected cells. Infected cells killed by CTLs undergo apoptosis, preventing further pathogen replication and release. CTLs are also capable of secreting cytokines to recruit more immune cells to the immune response. In contrast, activated CD4⁺ Th cells further differentiate into various effector subsets, which orchestrate the adaptive immune response by secreting cytokines to direct other immune cells appropriate for the pathogen (outlined in section 1.1.3).

1.1.3 *T helper cells and immune responses*

The Th1 and Th2 paradigm proposed by Mosmann and Coffman (1989) has been universally used to explain how hosts elicit different adaptive immune responses to eradicate invading pathogens. Classically, effector CD4⁺ T cells have been divided into two distinct lineages on the basis of their cytokine profiles: IFN- γ -producing Th1 cells or IL-4-producing Th2 cells. It is the most studied model for Th subset differentiation. In the last few years, there has been further subdivision of the CD4⁺ T cell effector phenotypes beyond that encompassed by the Th1/Th2 paradigm (Harrington *et al.*, 2006). The more recently defined effector CD4⁺ T cell subsets continue to be defined by the signature cytokines secreted, the transcription factors expressed and their surface phenotype. It is now apparent that CD4⁺ cells, upon encounter of specific antigen, are activated, expand and differentiate into various Th subsets expressing both distinct cytokine profiles and effector functions (Figure 1.1) (Korn *et al.*, 2009).

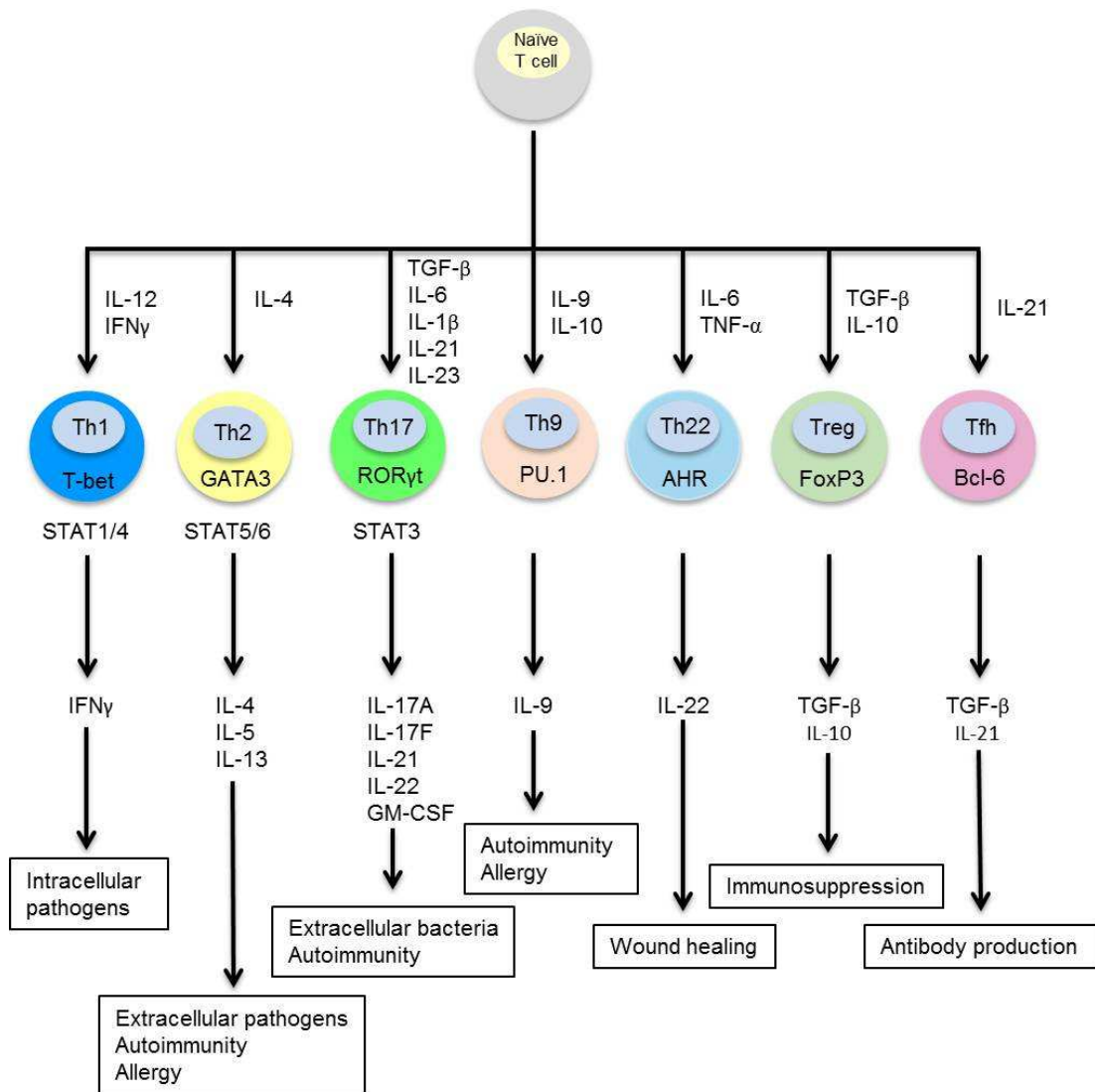


Figure 1.1. A simplified schematic tool of CD4⁺ T helper cell subsets. Activated naïve CD4⁺ T cells differentiate into various T helper (Th) cell subsets driven by specific cytokines and transcription factors. The effector Th subsets express signature cytokines, which regulate immune responses to specific types of pathogen. The Th cell subsets exhibit considerable lineage plasticity, which cannot be fully demonstrated in this figure. Adapted from Boniface *et al.* (2013).

The cytokine milieu present as a result of the early innate immune response is instrumental in the differentiation of CD4⁺ T cell subsets. In addition to cytokines and master transcription factors, Th cells utilise signalling molecules, such as signal transducer and activator of transcription factors (STATs), which couple cytokine receptors with gene expression (Adamson *et al.*, 2009). IL-12 and IFN- γ signalling

through predominantly STAT1, and to a lesser extent STAT4, up-regulates the master transcription factor “T-box expressed in T cells” (T-bet) (Afkarian *et al.*, 2002), which in turn drives expression of IFN- γ in Th1 cells. Th1 cells mediate cytotoxic T cell responses against intracellular pathogens, such as viruses and some bacteria, by the secretion of IFN- γ . If IL-4 and IL-2 are expressed in the local environment, STAT6 and STAT5 signalling activates trans-acting T-cell-specific transcription factor GATA-3 (GATA3), the master regulator of Th2 cells (Kaplan *et al.*, 1996). Th2 cells express the signature cytokines IL-4, IL-5 and IL-13, which activate B cells and drive Ab responses against extracellular pathogens such as parasitic worms. Th17 cells mediate acute inflammation at epithelial surfaces against extracellular bacteria and prokaryotes, and Treg cells inhibit T cell proliferation and autoimmune responses, dampening inflammatory responses (Wing *et al.*, 2006). The newer T cell subsets that have been established in mammals in the last few years include Tregs (Sakaguchi *et al.*, 1995), follicular helper T cells (Tfh) (Breitfeld *et al.*, 2000; Schaerli *et al.*, 2000), Th17 cells (Harrington *et al.*, 2005) and most recently, Th9 cells (Dardalhon *et al.*, 2008; Veldhoen *et al.*, 2008) and Th22 cells (Duhon *et al.*, 2009). These subsets of T cells have specific effector or regulatory functions and, by expressing different cytokines, mediate their influence on other cells during the immune response (Figure 1.1).

1.1.4 Cytokines

Cytokines are key messenger molecules in cell-to-cell communication within the innate and adaptive immune system. They consist of the ILs, IFNs, TNFs, colony-stimulating factors (CSFs), transforming growth factors (TGFs) and chemokines. During infection, a complex network of cytokines orchestrate the initiation,

maintenance and dampening of host defences. They determine the type of response and the effector mechanisms initiated to eliminate a pathogen. Further, transiently and locally expressed cytokines control the size and duration of the immune response, playing a crucial role in regulating inflammation and immunity. The other side of the coin, disproportionate or inadequate production of cytokines, contributes significantly to the pathology of a disease (Ingrao *et al.*, 2013). Typically, cytokines are secreted by a cell in response to stimulation and can exert their effect in an autocrine, paracrine or endocrine manner. Interleukins were originally defined as being produced and acting on white blood cells but are now recognised to have a much broader function and act on many different tissues. Interferons are named after their ability to ‘interfere’ with viral replication in a host cell, acting by stimulating innate cells to initiate anti-viral immune responses as well as recognising tumours. Tumour necrosis factors are pro-inflammatory and cause cell death by apoptosis. Colony stimulating factors direct haematopoietic stem cells to proliferate and differentiate. Transforming growth factors regulate cell division and survival and also have an anti-inflammatory function. Chemokines are so-called because of their ability to induce directed chemotaxis in nearby responsive cells. They play a vital role in directing lymphocytes, particularly phagocytes, during the immune response.

1.2 The avian immune system

The chicken differs from mammals in its repertoire of lymphoid tissues, cells and molecules. The avian immune system, while similar to its mammalian counterparts in many aspects, differs from mammalian models sufficiently that to understand host-pathogen interactions it is necessary to have a greater understanding of the elements

unique to birds. Birds and mammals evolved from a common ancestor over 300 million years ago but have continued to occupy the same biological niche and consequently encounter a similar range of pathogens. In broad terms, chickens respond to pathogens in much the same way as man; they too have developed innate and adaptive immune responses to overcome a diverse range of pathogens. Nevertheless, the chicken immune system has a number of unique features.

Similar to mammals, the chicken has both central and peripheral lymphoid tissues; however, the finer details differ. The central lymphoid tissues include the bone marrow, thymus and bursa of Fabricius (Figure 1.2). Peripheral lymphoid and myeloid cells originate from haematopoietic stem cells in the bone marrow. Immature chicken T cells migrate to the thymus to continue their development as in mammals. Chicken B cell development occurs in the bursa of Fabricius, which is strikingly different to B cell maturation in the bone marrow in mammals (section 1.2.2). Like mammals, the peripheral lymphoid tissues include the spleen as a key lymphoid organ and mucosal-associated lymphoid tissues, such as Harderian glands, caecal tonsils, Meckel's diverticulum and the oesophageal tonsil. However, the chicken lacks encapsulated lymph nodes and instead has diffuse mucosal-associated lymphoid tissues. Further, mammalian lymph node development is driven by two cytokines, the TNF superfamily members lymphotoxin α (LT- α) and LT- β (Alimzhanov *et al.*, 1997; De Togni *et al.*, 1994). These genes have not been found in the chicken genome to date (Kaiser, 2012), nor have their receptors, which may explain the lack of lymph nodes in the chicken. It is not yet clear where antigen presentation takes place in the chicken.

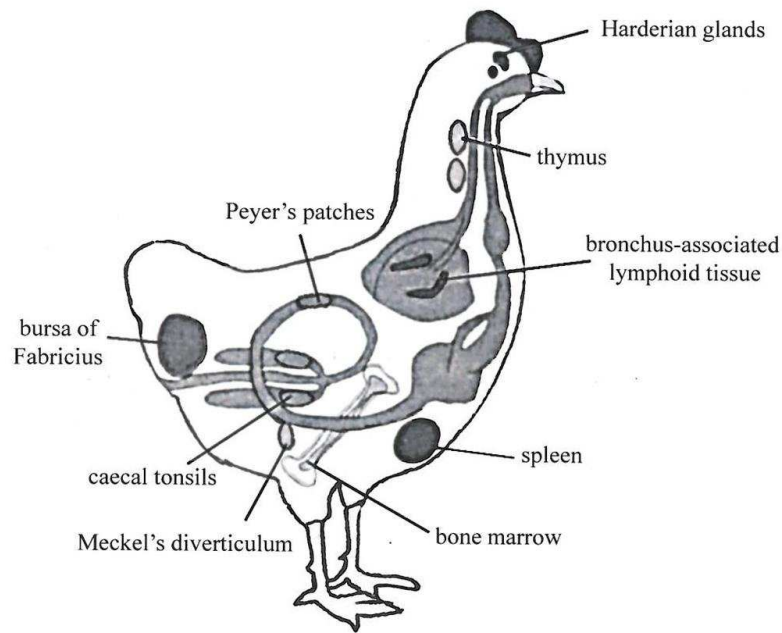


Figure 1.2. The lymphoid organs of the chicken. The central lymphoid organs include the bone marrow, thymus and bursa of Fabricius. The peripheral lymphoid tissues include the Harderian glands, spleen, bronchus-associated lymphoid tissues, Meckel's diverticulum, caecal tonsils and Peyer's patches.

1.2.1 The avian innate immune system

Many components of the human innate immune response do not have a recognised equivalent in the chicken or are not as well characterised. However, it is known that APCs recognise pathogen through PRRs, releasing cytokines and chemokines, which drive inflammatory responses and shape an appropriate adaptive response, as in mammals.

Wu *et al.* (2010) first described bone marrow-derived DCs (BMDCs) in a non-mammalian species. Identification of naturally derived DCs in the chicken has been attempted but progress is limited (reviewed by Kaiser, 2010). Relatively little is known about chicken DCs *in vivo* but it is assumed they function similarly to mammals and present antigen. BMDCs express PRRs, co-stimulatory molecules, MHC class II, cytokines, chemokines and chemokine receptors (Wu *et al.*, 2011; Wu

et al., 2010). Post-hatch, chicken macrophages differentiate from circulating bone marrow-derived precursors and complete their differentiation in peripheral tissues under the influence of CSFs (Garceau *et al.*, 2010). Phenotypically, avian macrophages appear to be similar to mammals. As with their mammalian counterparts, chicken macrophages play an important role in host defence against microbial infection. Chickens lack neutrophils; the functional equivalent in chicken is the heterophil (Harmon, 1998). Heterophils are also polymorphonuclear granulocytes that phagocytose microbial pathogens. Like neutrophils, heterophils migrate to sites of infection and form the first line of defence in early innate immune responses. Similar to neutrophil activation in mammals, activation of heterophils results in pro-inflammatory cytokine and chemokine release (Kogut *et al.*, 2005). The chicken also appears to lack functional eosinophils (Maxwell, 1987) and the eotaxins and eotaxin receptor, chemokines that control migration of eosinophils in mammals, are missing in the chicken genome (Kaiser, 2012). Eosinophils are recruited by Th2 immune responses against parasitic infections and are a key component of allergy in mammals.

DCs, macrophages and heterophils in the chicken express a different repertoire of PRRs compared to mammals. The TLRs are the most characterised of the PRRs in the chicken and are evolutionarily conserved compared to mammals. Ten different TLR genes have been identified in the chicken genome but the TLR repertoire is subtly different compared to mammals (Brownlie and Allan, 2011) (Table 1.1). Chicken TLR1, 2, 3, 4, 5 and 7 are orthologous to their mammalian counterparts but chickens lack TLR8 and 9. Chickens have two TLR1s (TLR1A and B) and two TLR2s (TLR2A and B) as a result of gene duplication. These variants dimerise to

form cell surface receptors which recognise the same PAMPs as human (hu) TLR2 (Higuchi *et al.*, 2008), which can dimerise with huTLR1, 6 and 10, thus suggesting chickens can recognise a similar range of PAMPs as mammals, although using a different TLR repertoire (Kaiser, 2010). Chickens also have two additional TLR genes, chicken (ch) TLR15 and chTLR21, which are absent in humans (Higgs *et al.*, 2006; Roach *et al.*, 2005). Conversely, huTLR9 and huTLR11 orthologues are absent in the chicken. Chicken TLR21 is a direct functional equivalent of huTLR9 (Brownlie *et al.*, 2009). Recent study suggests chTLR15 may have evolved as a broad-spectrum TLR, with a specific role in defence against avian pathogens (Nerren *et al.*, 2010). Finally, chTLR8 is a pseudogene (Philbin *et al.*, 2005). RLRs and NLRs are cytoplasmic sensors of intracellular pathogen invasion. The chicken has a reduced repertoire of RLRs and NLRs compared to mammals, only NOD1 (not NOD2) being present in the chicken genome (Kaiser, 2010).

Table 1.1. The human and chicken Toll-like receptor families.

Location and PAMP of TLR	Human	Chicken	Reference
Cell surface TLRs that recognise cell surface PAMP	TLR1/6/10	TLR1LA and TLR1LB	(Iqbal <i>et al.</i> , 2005a; Yilmaz <i>et al.</i> , 2005)
	TLR2	TLR2A and TLR2B	(Fukui <i>et al.</i> , 2001)
	TLR4	TLR4	(Leveque <i>et al.</i> , 2003)
	TLR5	TLR5	(Iqbal <i>et al.</i> , 2005b)
	TLR11	Not present	
	Not present	TLR15	(Higgs <i>et al.</i> , 2006; Nerren <i>et al.</i> , 2010)
Intracellular TLRs that recognise nucleic acid	TLR3	TLR3	(Iqbal <i>et al.</i> , 2005b)
	TLR7	TLR7	(Philbin <i>et al.</i> , 2005)
	TLR8	Pseudogene	(Philbin <i>et al.</i> , 2005)
	TLR9	TLR21 (functional equivalent)	(Brownlie <i>et al.</i> , 2009; Roach <i>et al.</i> , 2005)

Compared to mice and humans, chickens possess a higher number of $\gamma\delta$ T cells but with limited diversity of the variable regions of the TCR γ and δ genes (Su *et al.*, 1999). The highest numbers of $\gamma\delta$ T cells are seen in the gut, specifically the caecum, following infection in the chicken (Pieper *et al.*, 2011). Further, the number of $\gamma\delta$ T cells in the chicken increases with age, constituting up to 50% of the circulating T cells in an adult bird. This is in contrast to chicken NK cells of which there are comparatively fewer numbers in the blood compared to mammals, up to 1% compared to 10% in mammals (Cooper *et al.*, 2001; Gobel *et al.*, 2001). Chicken NK cells have been described in the embryonic spleen but the most frequent population is the intestinal epithelial lymphocyte population in the intestinal mucosa (Gobel *et al.*, 2001). The role of chicken NK cells during disease remains unclear and is reviewed by Rogers *et al.* (2008). In conclusion, the exact roles of NK and $\gamma\delta$ T cells in innate and adaptive immune responses in the chicken remain to be elucidated. Finally, the three complement pathways discussed in mammals (section 1.1.1) are present in the chicken, albeit with fewer components identified compared to mammals. When the avian innate immune response fails to neutralise an antigen, the adaptive immune response is triggered as in mammals.

1.2.2 The avian adaptive immune system

Chickens possess T and B cells, which generate a broadly similar adaptive immune response to infection, with a few prominent differences. B cells and Ab production are common to all vertebrates. B cells use their BCR as antigen receptors and differentiate into plasma cells capable of secreting antibodies. The Ig genes in birds, which generate a diverse repertoire of BCR specificities, undergo Ig gene rearrangement like their mammalian counterparts; however, BCR/Ab diversity in

birds is primarily generated by gene conversion, which usually occurs after initial recombination of the single VJ or VH genes and surface expression of the canonical BCR. The chicken light chain and heavy chain gene loci are encoded by single copies of V_L and J_L genes and V_H and J_H respectively, limiting diversity and minimising V(D)J rearrangement (Ratcliffe and Jacobsen, 1994; Reynaud *et al.*, 1985). This is in contrast to humans whose heavy and light chains are encoded by families of gene segments. Diversity of the Ig V region is instead generated by recombination with clusters of pseudogenes upstream of the heavy and light chain loci, which donate sequence to the functionally rearranged JV_L or VDJ_H genes (Reynaud *et al.*, 1987; Reynaud *et al.*, 1989).

The primary site of BCR repertoire generation in the chicken is the bursa of Fabricius, which is unique to birds. B cell development in chickens begins in haematopoietic tissues, such as the bone marrow, similarly to mammals but then migrate to the bursa of Fabricius, colonising in a single wave during embryonic development. Gene conversion primarily occurs here as well. Post-hatch, the bursa of Fabricius begins to involute as cells die and completely disappears by 6 months. Peripheral blood levels of B cells are maintained and these B cells are thought to derive from the bone marrow (Schat *et al.*, 2012). Chicken B cells express only three classes of Ab: IgM, IgA and IgY. The latter is the functional equivalent of IgG in mammals but does not undergo subclass switching. Birds do not have equivalents of mammalian IgD or IgE (Ratcliffe, 2006).

Chicken T cell precursors originate in the blood before migration to the thymus for further development. Here, the development of chicken T cells and the generation of TCR diversity through somatic DNA recombination are identical to the mechanisms

in mammals. Chicken T cell precursors differentiate into $\text{TCR}\gamma\delta^+$ and $\text{TCR}\alpha\beta^+$ T cells in the thymus. The antibody TCR1 (Sowder *et al.*, 1988) recognises $\text{TCR}\gamma\delta^+$ T cells, which constitute a large proportion of the total peripheral lymphocytes in birds. There are two subsets of $\alpha\beta$ T cells in the chicken, which express $\alpha\text{V}\beta 1$ or $\alpha\text{V}\beta 2$ and can be distinguished by the antibodies TCR2 and TCR3 respectively (Davidson and Boyd, 1992). Similarly to their mammalian counterparts, thymic chicken $\alpha\beta$ T cells are initially double-positive $\text{CD4}^+\text{CD8}^+$ cells, which undergo positive selection to become single-positive CD4^+ or CD8^+ T cells. Following selection, naïve T cells enter the circulation and migrate to the spleen. It is predicted that antigen presentation and signalling through the chicken TCR complex is similar to mammals based on the strong conservation of intracellular signalling motifs but this has not been fully investigated in the chicken. The MHC, genetic locus for the MHC class I and II molecules, of the chicken has a reduced number of genes compared to mammals and differs in its genomic organisation (Kaufman *et al.*, 1999). Despite the chicken MHC being smaller than the mammalian MHC, nearly all of the chicken MHC genes have human MHC counterparts. There are many examples in the chicken of disease associations with the MHC (Bacon, 1987), e.g. chicken lines resistant and susceptible to MDV (Briles *et al.*, 1977) and the concept of the “minimal essential MHC of the chicken” provides a molecular basis for this. The multigene families that make up the mammalian MHC confer protection against most pathogens, to a greater or lesser extent, whereas the smaller, simpler chicken MHC confers either resistance or susceptibility to a pathogen (Kaufman and Salomonsen, 1997).

Knowledge of the existence of the chicken equivalents of the mammalian CD4⁺ T cell subsets is slowly advancing. Despite the Th1/Th2 paradigm being demonstrated almost a decade ago in the chicken (Degen *et al.*, 2005), and the more recent identification of Tregs (Shanmugasundaram and Selvaraj, 2010; 2011), the presence of the newer Th subsets (Th17, Th9, Th22 and Tfh) has yet to be confirmed or dismissed in the chicken (Kaiser, 2010). Including the work in this thesis, all of the cytokines crucial to driving mammalian Th lineage development have now been identified in the chicken (Kaiser, 2012). However, the master regulators of transcription for Th17 and Tregs, retinoic-acid-related (RAR) orphan receptor gamma t (ROR γ t) and Forkhead box P3 (FoxP3) respectively, have not been identified in the chicken. Also, during a Th2 response in chickens, IL-4 and IL-13 are expressed, similarly to their mammalian counterparts (Degen *et al.*, 2005; Powell *et al.*, 2009) but IL-5 expression is switched off. In mammals, IgE production is dependent on IL-5 expression by Th2 cells and as discussed previously, IgE is not produced in birds. Given these significant differences between the mammalian and avian immune systems, and the importance of Th17 immune responses in mammals, it is fundamental to better understand if the Th17 system exists in chickens, if it is similar to mammals, and, if not, what the major differences are.

1.2.3 Avian cytokines

The repertoire of cytokines in mammals is extensive and considerably larger than that currently known in the chicken, consistent with the trend of minimalism in the avian immune system compared to the mammalian immune system (Kaiser, 2010). So far, the genes for 27 ILs, all the TGFs, all the CSFs, 10 type I IFNs, IFN- γ , a single IFN- λ , 12 TNFs and 24 chemokines have been identified (Kaiser, 2010). The

availability of the chicken genome sequence (Hillier *et al.*, 2004) has aided in the discovery of new cytokine genes. However, progress in definitive identification is hindered by the lack of reagents and low sequence homology with mammalian orthologues. Typically, chicken cytokines have relatively low amino acid (aa) identities (18-40%) with human homologues but generally effector functions appear to be conserved. To date, the driving cytokines of Th17 cells, namely TGF- β 4, IL-6, IL-1 β , and IL-21, have been identified in the chicken. Further, it is known that the effector cytokines IL-17A, IL-17F, IL-21 and IL-22 are expressed in the chicken (Kim *et al.*, 2012a; Kim *et al.*, 2012b; Min and Lillehoj, 2002; Rothwell *et al.*, 2012). Interestingly, until now, IL-23 and its receptor were crucially missing from the chicken genome (section 1.3).

1.3 Th17 immune responses in mammals

Th17 cells are one of the newer T helper cell subsets to be described in mammals. Aggarwal *et al.* (2003) first described the idea of an IL-23/IL-17 pro-inflammatory axis; however, it was Harrington *et al.* (2005) who described the Th17 cell, a distinct CD4⁺ T cell subset critically responsible for the production of IL-17. Th17 cells are characterised by the production of the cytokines IL-17A, IL-17F, IL-21 and IL-22 (Korn *et al.*, 2009), granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN- γ . Differentiated Th17 cells express the IL-23 receptor (IL-23R) (McGeachy *et al.*, 2009). ROR γ t is only expressed by Th17 cells, and no other Th cell subsets, and its loss profoundly impairs the functionality of Th17 cells, which led to its characterisation as the master regulator of Th17 transcription (Ivanov *et al.*, 2006).

Critically, the Th17 subset represents an arm of the adaptive immune system that functions to clear specific types of pathogen not effectively neutralised by Th1 or Th2 immune responses (Korn *et al.*, 2009). Current data imply a specialised evolutionary function of Th17 cells to promote protective immunity at mucosal sites against a variety of pathogens (Kolls and Khader, 2010). Th17 is widely considered to be the main arsenal in host defence against extracellular bacteria but recent studies also suggest that the Th17 subset has evolved to confer protective immunity against intracellular pathogens (Khader and Gopal, 2010; Peck and Mellins, 2010). This duality is achieved by regulating and bridging the innate and adaptive immune responses (Kolls and Khader, 2010; Stockinger and Veldhoen, 2007). For example, IL-17 stimulates the mobilisation and *de novo* generation of neutrophils by granulocyte-colony stimulating factor (G-CSF) (Fossiez *et al.*, 1998). Th17 cells can also promote multiple aspects of humoral immunity, including germinal centre formation and antibody isotype switching, as well as vaccine-induced immunity by aiding Th1 cell recruitment (Khader *et al.*, 2007; McAleer and Kolls, 2011; Mitsdoerffer *et al.*, 2010). The Th17 subset provides a vital host defence function but it can also promote severe chronic inflammation. Experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis (MS) in humans, was pivotal in unravelling the role of Th17 cells in autoimmune disease (Figure 1.3). Ablation of EAE disease was seen in mice deficient in the p40 subunit of IL-12 (Leonard *et al.*, 1995); however, mice deficient in IFN- γ , the principle cytokine produced by Th1 cells, are susceptible to EAE (Ferber *et al.*, 1996). With the discovery of IL-23, which is made up of p19 and p40, the latter of which it shares with IL-12 (Aggarwal *et al.*, 2003), Cua *et al.* (2003) were able to show that mice

deficient in the p19 subunit of IL-23 were protected against EAE disease development and that IL-23, not IL-12, was responsible for EAE pathogenesis.

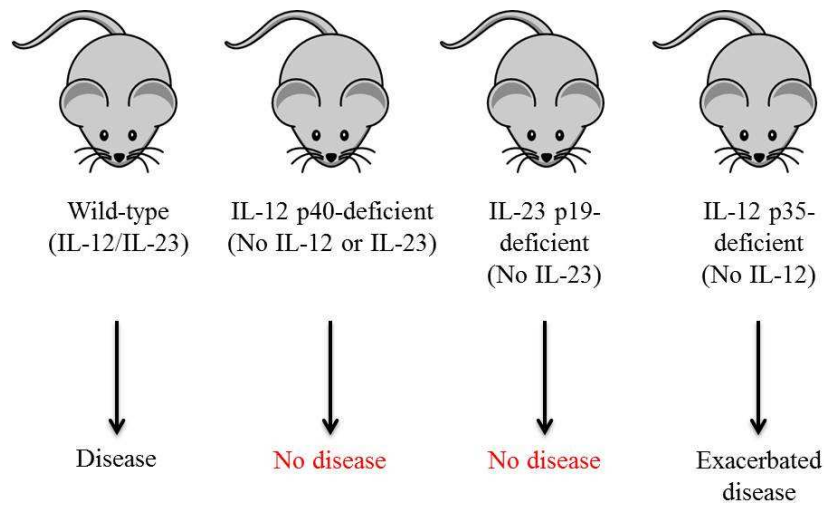


Figure 1.3. The pathogenic role for IL-23, not IL-12, in mouse models of autoimmunity. EAE disease development requires IL-23, but not IL-12. Compared with wild-type susceptible mice, mice deficient for IL-23 (IL-23 p19^{-/-}) and both IL-23 and IL-12 (IL-12 p40^{-/-}) failed to develop disease after antigenic challenge, whereas mice deficient for IL-12 (IL-12 p35^{-/-}) developed more severe disease (Harrington *et al.*, 2006).

1.3.1 Differentiation

The description of Th17 cells has caused a major paradigm shift in our understanding of T cell biology and immune regulation. The identification of this novel antigen-dependent T cell subset has removed the dichotomous classification of Th1 and Th2, which had remained established for over 20 years. A further unexpected finding was the concept of T cell plasticity. Th17 cells are highly unstable in many experimental systems (Lee *et al.*, 2009) and these initial observations raised the question of whether or not Th17 cells represent a distinct T cell subset. It is generally accepted that because Th17 cells develop independently of STAT1, T-bet, STAT4 and STAT6 and are the only Th subset to express RORγt that they are a distinct Th cell lineage (Harrington *et al.*, 2005; Park *et al.*, 2005). Networks of transcription factors regulate cytokine and effector gene expression and direct Th cell differentiation and

expression of effector phenotypes. The transcription factors STAT3, ROR γ t and ROR α (Yang *et al.*, 2008a; Yang *et al.*, 2008b) all play a role in Th17 differentiation, as does activation of aryl hydrocarbon receptor (Ahr) but it remains unclear what features of Th17 differentiation are regulated by this latter transcription factor (Stockinger *et al.*, 2011). The developmental pathway of naïve Th cells is determined at least in part by the cytokine milieu present when they are activated. The inflammatory cytokines TGF- β , IL-6, IL-1 β (Veldhoen *et al.*, 2006), IL-21 (Korn *et al.*, 2007; Zhou *et al.*, 2007) and IL-23 (Volpe *et al.*, 2008) are required for the development of Th17 cells in mammals (Figure 1.4). IL-23 acts as a growth and stabilisation factor for Th17 cells (McGeachy *et al.*, 2007; McGeachy *et al.*, 2009; Veldhoen *et al.*, 2006).

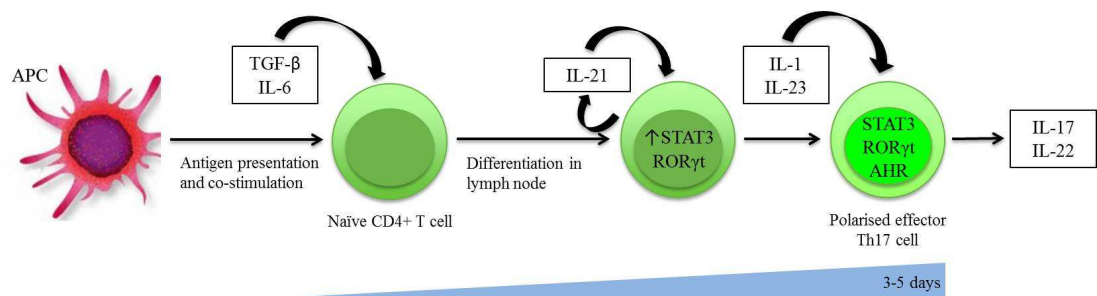


Figure 1.4. Th17 cell differentiation. APCs present antigen using MHC molecules in the presence of TGF- β , IL-6, IL-21, IL-23 and IL-1 β . This initial activation up-regulates STAT3 and ROR γ t expression to enhance IL-23 responsiveness and IL-17 production. Adapted from Cua and Tato (2010).

Despite Th17 differentiation being extensively investigated, the exact *in vivo* differentiation requirements remain incompletely defined. T cell activation in the presence of TGF- β and IL-6 primes the initial differentiation of naïve CD4⁺ T cells to IL-17-producing Th17 cells. It is generally agreed that TGF- β , IL-6 and IL-1 β are required. Confusion arises over whether the requirement of TGF- β because of differences between mice and humans and the requirement of TGF- β to induce

Tregs. It is now accepted that TGF- β is required but it is difficult to prove since removing it from tissue culture is extremely hard due to its expression from so many different cell types. The effect of TGF- β in a Th17 context is dependent on its concentration in the cytokine milieu: low levels promote Th17 differentiation by inhibiting T-bet whereas higher levels inhibit IL-23R expression and hinder Th17 differentiation. Zhou *et al.* (2008) showed that lower levels of TGF- β with IL-6 overcame FoxP3-mediated repression of ROR γ t to drive Th17 differentiation. FoxP3 is the master regulator of transcription of naturally arising CD4⁺ regulatory T cells. Therefore, the concentration of TGF- β in the local cytokine milieu regulates Treg versus Th17 cell development.

Veldhoen *et al.* (2006) showed that for Th17 cell differentiation, TGF- β and IL-6 drive the coordinated activation of the transcription factors *Caenorhabditis elegans* SMA (SMA) and mothers against decapentaplegic (MAD) homolog 3 (SMAD3) and STAT3 to induce the transcription factors ROR α and ROR γ t, which are necessary for Th17 cell differentiation. Th17 cell activation requires full activation of STAT3 by phosphorylation and IL-6 and IL-1 β both play roles in this process: IL-6 via Janus kinase 2 (JAK2) and IL-1 β via IL-1 receptor-associated kinase 1 (IRAK1) (Ivanov *et al.*, 2006). STAT3, in turn, drives expression of ROR γ t, IL-17, IL-21 and IL-23R (Durant *et al.*, 2010; Wei *et al.*, 2007). The expression of IL-21, acting synergistically with TGF- β , is subsequently important in the induction and amplification of Th17 cells. IL-21 acts via an autocrine loop, inducing its own gene transcription in a STAT3-dependent manner, further enhancing expression of IL-21 and IL-23R (Nurieva *et al.*, 2007; Zhou *et al.*, 2007). IL-23 is now understood to be required for full maturation and inflammatory function of Th17 cells. Addition of IL-

23 also markedly enhances IL-23R gene expression by IL-23R⁺RORγt⁺ Th17 cells, providing amplification of its own signalling via its own receptor. IL-23/IL-23R signalling induces RORγt, further up-regulating IL-23R expression. Therefore, IL-23 is essential for full maturation of Th17 cells, including effector differentiation, acquisition of inflammatory phenotype and stabilisation of the lineage (Zuniga *et al.*, 2013). All of the cytokine pathways involved in Th17 cell differentiation result in up-regulation of RORγt expression, the master regulator of Th17 differentiation.

In addition to the positive effects of cytokines on Th17 development, they are also able to exert an inhibitory effect. Specifically, Th17 development is inhibited by the Th1 and Th2 cytokines, IFN-γ and IL-4, as well as STAT1-activating cytokines, e.g. type I IFNs and IL-27. Th17 generation is also suppressed by IL-2, which activates STAT5, which in turn competitively binds at the same locus as STAT3 (Yang *et al.*, 2011). Further, TGF-β suppresses the transcription factors T-bet and GATA3, inhibiting CD4⁺ T cells from adopting Th1 or Th2 fates (Basu *et al.*, 2013; Harrington *et al.*, 2005). However, the developmental requirement of Th17 cells for TGF-β is shared with Tregs (Bettelli *et al.*, 2006). Lee *et al.* (2009) have shown that Th17 cells can deviate towards a Th1 phenotype by expressing IFN-γ in response to further IL-23 or IL-12 signalling. This plasticity of Th17 cells initially clouded the issue of whether they were a genuine additional Th cell subset and consequently precisely what preserves phenotypic commitment of CD4⁺ T cells is currently intensely investigated. There is considerable heterogeneity and plasticity amongst the defined CD4⁺ T cell subsets, which has been extensively reviewed (Murphy and Stockinger, 2010; Nakayamada *et al.*, 2012; O'Shea and Paul, 2010). TGF-β induces both Foxp3 and RORγt in naïve T cells but Foxp3 is dominant until IL-6 is present

(Zhou *et al.*, 2008), indicating an inflammatory environment tips the balance between iTreg and Th17 differentiation. It is now evident that cytokine expression is also not as stable as initially thought. Th17 cells are able to acquire IFN- γ -producing potential and simultaneously produce IL-17 and IFN- γ (Wilson *et al.*, 2007). Further, Th17 cells are able to extinguish expression of their signature cytokine and become selective IFN- γ -producers (Lee *et al.*, 2009).

1.3.2 Effector cytokines and functions

Th17 cells can communicate with a broad range of cell types by inducing the production of pro-inflammatory cytokines and chemokines, resulting in protection from invading microbial pathogens (Korn *et al.*, 2009). These effects are mediated by Th17 production of effector cytokines such as IL-17A, IL-17F, IL-21 and IL-22. In addition, Th17 cells produce the non-Th17-specific cytokines GM-CSF, IFN- γ and IL-10 (El-Behi *et al.*, 2011; Ivanov *et al.*, 2006; McGeachy *et al.*, 2007) widening the spectrum of Th17 phenotypes by varying cytokine production and effector function.

IL-17A and IL-17F belong to the IL-17 family of cytokines, which has four other members, designated IL-17A-F. IL-17A and IL-17F are produced by Th17 cells, IL-17E, also known as IL-25, is produced by Th2 cells, whereas the other family members, IL-17B, IL-17C, IL-17D, are produced by non-T cell sources (Ouyang *et al.*, 2008). IL-17A and IL-17F can form homo or hetero-dimers, therefore giving rise to 3 forms of IL-17: IL-17A/A, IL-17F/F and IL-17A/F, which potentially have different biological activities. IL-17A/A appears to be most potent of the three in tissue inflammation. However, IL-17A/F is produced in the largest amounts by polarised Th17 cells (Liang *et al.*, 2007). IL-17A, IL-17F and IL-17A/F heterodimers

signal through the receptor subunits IL-17RA and IL-17RC, which together form a heterodimeric complex to transduce downstream signalling (Kuestner *et al.*, 2007; Wright *et al.*, 2008; Yao *et al.*, 1995a). IL-17A and IL-17F cellular expression is tightly controlled. However, IL-17RA is expressed ubiquitously (Ishigame *et al.*, 2009; Yao *et al.*, 1995a). In contrast to IL-17RA expression, IL-17RC is preferentially but lowly expressed on non-haematopoietic cells (Kuestner *et al.*, 2007). This differential expression may provide a mechanism for the tissue-specific functions of IL-17.

Th17 cellular responses do not explain early IL-17-mediated immunity, which is vital during stress responses and host defence. Innate sources of IL-17 include $\gamma\delta$ T cells (Ness-Schwickerath *et al.*, 2010), natural killer T (NKT) cells (Michel *et al.*, 2008), LT α i cells (Takatori *et al.*, 2009), NK cells (Passos *et al.*, 2010) macrophages, DCs, neutrophils, mast cells and myeloid cells (Figure 1.5) (reviewed by Cua and Tato, 2010). Thus IL-17A and IL-17F are produced by cells of the innate and adaptive immune systems, and activation of their receptors results in innate immune signalling, acting as a bridging function between innate and adaptive immunity. The exact role of the innate cell populations expressing IL-17 in directing T cell effector and memory responses remains to be elucidated. However, it is notable that most IL-17-producing cells are found in tissues which protect the host from the environment, e.g. lungs, intestinal mucosa and skin (Lochner *et al.*, 2008).

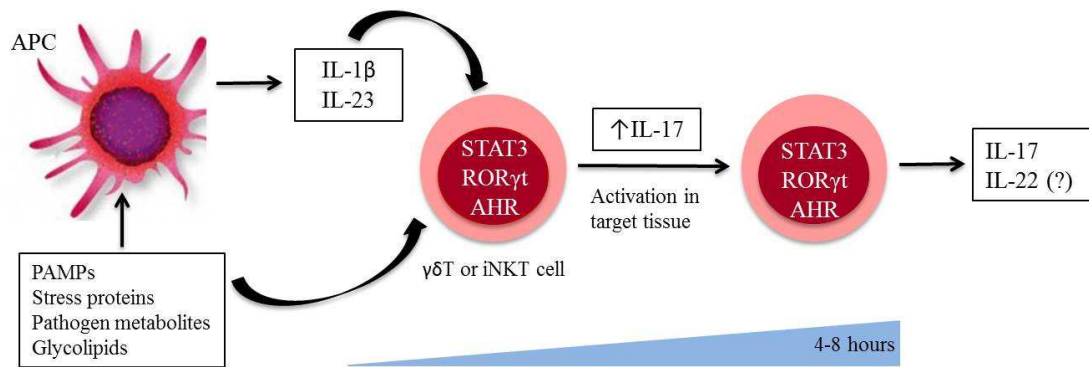


Figure 1.5. Activation of innate cells to produce IL-17. Innate cells are activated by cytokines such as IL-1 β and IL-23, alone or in combination with PAMPs or through their TCR when expressed. These cells constitutively express transcriptional regulators for IL-17 production so can produce IL-17 within hours of stimulation. Adapted from Cua and Tato (2010).

Both IL-17A and IL-17F are pleiotropic cytokines, acting on a broad range of cell types to induce the expression of pro-inflammatory cytokines (IL-6, TNF- α , IL-1 β , GM-CSF and G-CSF) (Fossiez *et al.*, 1996; Jones and Chan, 2002; Jovanovic *et al.*, 1998) and chemokines (CXCL1, CXCL8 and CXCL10) (Witowski *et al.*, 2000), which potentially mediate IL-17 function by attracting neutrophils (Laan *et al.*, 1999), metalloproteinases (Prause *et al.*, 2004) and anti-microbial peptides such as β -defensins (Kao *et al.*, 2004) *in vivo*. Accordingly, IL-17A and IL-17F are key cytokines in the recruitment, activation and migration of neutrophils to the site of inflammation (Aggarwal and Gurney, 2002).

IL-21 is a member of the IL-2 family of cytokines and the IL-21R is mainly expressed on B cells but also on T cells and other myeloid cells (Parrish-Novak *et al.*, 2000). It signals via the common γ -chain-receptor and an IL-21-specific receptor protein (IL-21R) to activate the JAK-STAT pathway, preferentially phosphorylating JAK1 and JAK3 to further activate STAT3 (Asao *et al.*, 2001; Habib *et al.*, 2002). It has pleiotropic effects on both innate and adaptive immune responses and its role in Th17 immunity is discussed in section 1.3.1. In addition to the differentiation of

Th17 cells, IL-21 stimulates proliferation and differentiation of CD8⁺ T cells (Kasaian *et al.*, 2002), B cell differentiation and antibody class switching (Ozaki *et al.*, 2004), and NK cell survival and cytotoxicity (Skak *et al.*, 2008).

IL-22 is a member of the IL-10 family of cytokines (Dumoutier *et al.*, 2000). Originally designated a Th1-associated cytokine, it is most highly expressed by Th17 cells in mice (Liang *et al.*, 2006) and is additionally expressed by activated T cells (CD4⁺, CD8⁺, $\gamma\delta$ and NKT cells), LTi and NK cells (Crellin *et al.*, 2010; Doisne *et al.*, 2011; Liu *et al.*, 2011; Martin *et al.*, 2009; Wolk *et al.*, 2002). IL-22 signals through the heterodimeric receptor complex of the specific IL-22R and IL-10R2 (Kotenko *et al.*, 2001; Xie *et al.*, 2000), with signal transduction through IL-22R activating STAT3. Expression of the IL-22R receptor chain is indicative of an IL-22 target cell and expression is mostly found in cells from the skin, digestive and respiratory tracts (Trivella *et al.*, 2010). Unlike many cytokines, IL-22 does not function to communicate between immune cells (Wolk *et al.*, 2010) and acts exclusively on non-haematopoietic cells (Sonnenberg *et al.*, 2011). *In vivo*, IL-22 expression is critically dependent on IL-23 (Zheng *et al.*, 2007) and *in vitro* production is optimal when IL-23 is present (Liang *et al.*, 2006).

IL-22 is important in anti-microbial defence, regeneration and protection against damage. Not only does it induce the expression of anti-microbial peptides, strengthening host defence directly but it also modulates tissue responses during inflammation and aids in tissue repair (Hirota *et al.*, 2010). In the context of chronic inflammatory disorders, IL-22 may be either protective or highly pathogenic (Wolk *et al.*, 2010). Its complex effects depend on the inflammatory context, e.g. on the duration and amount of IL-22 present, the surrounding cytokine milieu and the

tissues involved (Zenewicz and Flavell, 2011). Overall, IL-22 functions to promote barrier immunity by limiting bacterial replication.

GM-CSF was originally discovered as a haematopoietic growth factor, capable of stimulating the proliferation of myeloid cells from bone-marrow progenitors (Burgess *et al.*, 1977). It is a member of the common β -chain receptor family of haematopoietic cytokines and signals through a heterodimeric receptor composed of the ligand-specific CSF2R α and a signal transducing β -chain, CSF2R β (Gearing *et al.*, 1989; Hayashida *et al.*, 1990). GM-CSF is now recognised to have a huge range of functions. On mature haematopoietic cells, GM-CSF enhances antigen presentation, polarisation of macrophages and induction of complement- and antibody-mediated phagocytosis as well as priming neutrophils. In addition, it is produced by a wide variety of cells including T cells, B cells, macrophages, endothelial cells and fibroblasts (reviewed by Fleetwood *et al.*, 2005). It was first suggested by Hamilton *et al.* (1980) that GM-CSF should be viewed as a pro-inflammatory cytokine and not simply a proliferation factor for macrophages. Codarri *et al.* (2011) and El-Behi *et al.* (2011) have all shown GM-CSF to be another Th17 cytokine, which contributes to the Th17 effector phenotype based on an EAE model, the mouse model for MS. GM-CSF, induced by IL-23, is indispensable for Th17-mediated EAE pathogenesis; GM-CSF-deficient mice are highly resistant to EAE (Ghoreschi *et al.*, 2010). GM-CSF up-regulates IL-6 and IL-23 expression, further enhancing GM-CSF production in a positive feedback loop and promoting *de novo* generation of Th17 cells (El-Behi *et al.*, 2011). There are conflicting data as to whether the master regulator, ROR γ t, is the transcription factor, which drives GM-CSF production in Th17 cells (Codarri *et al.*, 2011; El-Behi *et al.*, 2011).

IFN- γ is the signature cytokine expressed during a Th1 immune response and is under the control of the master transcription factor T-bet. It is a type II IFN that is critical in innate and adaptive immunity. It is predominantly expressed by APCs, NK cells, Th1 cells and CD8⁺ CTLs (Schroder *et al.*, 2004). IFN- γ up-regulates the MHC class I and II presentation pathways on APCs, therefore promoting activation of CD8⁺ and CD4⁺ T cells. As a major product of Th1 cells, it skews the immune response towards a Th1 phenotype promoting cell-mediated immunity via activation of NK cells and macrophages. Th17 cells generated *in vivo* often co-produce IFN- γ (Abromson-Leeman *et al.*, 2009; Acosta-Rodriguez *et al.*, 2007a; Ivanov *et al.*, 2006). Until recently, there was much debate over whether these IL-17-IFN- γ double-producing T cells were derived from Th1 or Th17 cells. Hirota *et al.* (2011) settled the debate by generating a mouse that allowed for identification of all cells that have ever expressed IL-17, and demonstrated that the IL-17-IFN- γ double-producing T cells were exclusively derived from Th17 cells. It has not been shown conclusively whether the production of IFN- γ by Th17 cells is protective or pathogenic (Peters *et al.*, 2011) or what is the functional advantage for Th17 cells in demonstrating such plasticity.

IL-10 was first described as cytokine synthesis inhibitory factor in mice (Fiorentino *et al.*, 1989) and is almost certainly the most important anti-inflammatory cytokine in mammals. IL-10 is expressed by a huge number of cells including APCs, T and B cells, granulocytes, epithelial cells, keratinocytes and mast cells. It limits secretion of pro-inflammatory cytokines, deactivates macrophages and controls proliferation of many cell types including macrophages, T and B cells (reviewed by Moore *et al.*, 2001). IL-10 is the primary anti-inflammatory cytokine of the immune system that

protects against excessive immune responses and tissue damage, thus maintaining immune homeostasis. To exert its actions, IL-10 dimerises and binds to its tetrameric cytokine receptor made up of two molecules of IL-10R1 and two molecules of IL-10R2 (Kotenko *et al.*, 1997; Liu *et al.*, 1994). Th17 cells produce variable amounts of IL-10 (McGeachy *et al.*, 2007) as well as expressing the IL-10R α (Huber *et al.*, 2011). IL-10-mediated Th17 suppression is brought about by the regulatory functions of Tregs but also by direct inhibition through IL-10 signalling (Huber *et al.*, 2011). It is hypothesised that Th17 cells may produce IL-10 in order to restrain themselves and prevent unnecessary tissue destruction (Peters *et al.*, 2011).

1.3.3 Pathogenic effects

Before the discovery of Th17 cells, experimental data from autoimmune disease studies could not be explained solely by the Th1/Th2 paradigm. The discovery of IL-23 (Aggarwal *et al.*, 2003), and its vital role in the development of central nervous system autoimmune inflammation (Cua *et al.*, 2003), was the first step in understanding the pathogenesis of Th17 immune responses in autoimmunity. Since then, Th17 cells and their cytokines have been linked to several autoimmune and inflammatory diseases such as rheumatoid arthritis (RA), MS, psoriasis, systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), asthma and allergy (reviewed by Hu *et al.* (2011); Maddur *et al.* (2012); Ouyang *et al.* (2008); Qu *et al.* (2013)). The exact pathogenic mechanisms of autoimmune disease are poorly understood but the inflammatory cascades triggered by Th17 cells and the effector cytokines they express clearly have a role in the pathogenesis.

RA is a chronic inflammatory disease of the joints and is one of the most common autoimmune-related diseases. Increased levels of IL-17A are seen in sera and synovial fluid and increased levels of IL-22 and IL-23 are seen in the synovium of RA patients (Ikeuchi *et al.*, 2005; Kim *et al.*, 2007b; Ziolkowska *et al.*, 2000). Interestingly, the increase in IL-17A and IL-23 is specific for RA and not osteoarthritis (Honorati *et al.*, 2001; Kim *et al.*, 2007a). These Th17 cytokines stimulate various cell types to produce cytokines, chemokines and destructive mediators which drive inflammation in the joints.

As previously mentioned, IL-23 is critical for the induction of EAE in mice (Cua *et al.*, 2003). In addition, in patients with MS, IL-17A expression is correlated with disease activity. IL-17A⁺ T cells, astrocytes and oligodendrocytes are increased in active, rather than inactive, MS lesions (Tzartos *et al.*, 2008). Recently, Codarri *et al.*, 2011) showed a critical non-redundant role of Th17-produced GM-CSF in the pathogenesis of EAE. GM-CSF drives APCs to secrete IL-23 in a positive feedback-loop and absence of GM-CSF confers resistance to EAE in mice. However, the exact pathogenic role of Th17 cells, or their effector cytokines, in EAE/MS is not well understood.

Psoriasis is a chronic immune-mediated skin disease in which therapeutic antibodies against IL-17A have shown promising results in late-stage clinical trials (Leonardi *et al.*, 2012). Th17 cells are present in psoriatic lesions (Lowes *et al.*, 2008) and IL-22 expressed by infiltrating lymphocytes may have a role in the pathogenesis (Boniface *et al.*, 2007). In terms of genetics, polymorphisms in IL-12/23 p40 and IL-23R have been associated with psoriasis (Nair *et al.*, 2009).

In asthmatic patients, the number of Th17 cells in peripheral blood is enhanced (Li *et al.*, 2013). IL-17 production induces a broad tissue response, including neutrophil trafficking to the site of inflammation and activation of airway smooth muscle cells and epithelial cells to produce pro-inflammatory mediators (Korn *et al.*, 2009; Kudo *et al.*, 2012). The exact role of Th17 cells in asthma remains elusive despite the important roles of IL-17A and IL-17F in airway neutrophilia in a mouse model of asthma (Hellings *et al.*, 2003).

There has been much research in mammals investigating Th17 immune responses, from the differentiation factors required, the effector cytokines expressed and the cell types upon which they act, to the detrimental effects of failure to control Th17 cells. To begin to establish if Th17 cells have a role in the avian immune system, the cytokine IL-23 and its receptor need to be identified and characterised in the chicken.

1.4 Th17 immune responses in birds

The Th17 cell subset has not yet been described in birds. Several components of Th17 immune responses have been identified in the chicken but a few key molecules are still currently “missing” from the chicken genome (Table 1.2), including IL-23, ROR γ t and IL-23R.

Table 1.2. Th17 immune response components in mammals and chicken.

Mammalian Th17 molecule	Identified in chicken
TGF- β	TGF- β 4 is orthologous to mammalian TGF- β 1 (Jakowlew <i>et al.</i> , 1997)
IL-1 β	(Weining <i>et al.</i> , 1998)
IL-6	(Schneider <i>et al.</i> , 2001)
IL-21	(Rothwell <i>et al.</i> , 2012)
IL-23	-
ROR γ t	-
STAT3	(Wang <i>et al.</i> , 2007)
IL-23R	-
IL-17A	(Min and Lillehoj, 2002)
IL-17F	(Kim <i>et al.</i> , 2012b)
IL-22	(Kim <i>et al.</i> , 2012a)
GM-CSF	(Avery <i>et al.</i> , 2004)

1.4.1 IL-23

IL-23 is a heterodimeric cytokine composed of a p19 subunit paired with a p40 subunit, which is shared with IL-12. It is a member of the IL-12 family of cytokines (Oppmann *et al.*, 2000). The p19 subunit, similarly to the p35 subunit of IL-12, has a four-alpha-helical bundle and is linked to the p40 protein by disulphide bonds. IL-23 and IL-12 are constitutively expressed as a binary complex of the p19 or p35 subunits respectively and p40 (Lupardus and Garcia, 2008). This ensures the formation of biologically active protein (Langrish *et al.*, 2004). IL-23 is mainly produced by cells of the innate immune system, such as DCs and tissue-resident macrophages, in peripheral tissues such as the skin, intestinal mucosa and lungs (Basso *et al.*, 2009). Production is stimulated by activation of TLR ligands such as LPS, peptidoglycan, CpG deoxyribonucleic acid (DNA) and polyinosinic:polycytidylic acid (poly I:C) (Kastelein *et al.*, 2007). Enhanced IL-23

production from APCs is also seen with CD40-CD40L interaction. However, the regulation of IL-23 expression remains incompletely understood.

The full differentiation and maintenance of Th17 cells is dependent on IL-23 (Korn *et al.*, 2009; McGeachy *et al.*, 2009). However, after identification of the differentiation factors required for Th17 cells, it became clear that IL-23 was not involved in the initial differentiation stage (Korn *et al.*, 2009) but is fundamental to maintain Th17 cells *in vitro* and *in vivo* (Chen *et al.*, 2011). Naïve T cells do not express IL-23R; ROR γ t expression is required to control expression of IL-23R on newly primed T cells. This in turn expands their responsiveness to IL-23 by expression of IL-23R on activated/memory T cells (Aggarwal *et al.*, 2003; Kastelein *et al.*, 2007; Kolis and Khader, 2010), allowing Th17 cells to become responsive to IL-23 and subsequently allows IL-23 to serve as a survival factor for committed Th17 cells (Veldhoen *et al.*, 2006).

The supposition that the early role of mammalian IL-23 is only in the generation of Th17 cells has proven exceedingly over simplified, given the essential role for IL-23 in several models of autoimmunity such as psoriasis, IBD and EAE has been established (Figure 1.6) (Becher *et al.*, 2002; Hedrick *et al.*, 2009; Uhlig *et al.*, 2006). It is clear that the pathogenic mechanism of IL-23 is not limited to Th17 cells and their expression of IL-17A. Multiple IL-23-responsive cell types, which express pro-inflammatory cytokines, are emerging. $\gamma\delta$ T cells constitutively express the IL-23R (Sutton *et al.*, 2009) and the epithelium of the skin and gut are enriched with these cells. Group 3 ILCs (ILC3s) respond to IL-23 (Buonocore *et al.*, 2010) and IL-23R signalling drives expression of IFN- γ and IL-17.

IL-23 may play a role in an early, immediate tissue inflammatory response, through the action of innate immune cells such as $\gamma\delta$ T cells and ILC3s, as well as later on in an antigen-dependent fashion through the action of memory T cells (Croxford *et al.*, 2012). IL-23 p19 has been identified in many mammalian species (Chen *et al.*, 2009; Gossner *et al.*, 2012; Kokuho *et al.*, 2012; Tompkins *et al.*, 2010) and also in a non-mammalian species, the zebrafish (*Danio rerio*) (Holt *et al.*, 2011) but to date, it has not been identified in an avian species.

The receptor for IL-23 is made up of two subunits, IL-12R β 1 and IL-23R, and is a member of the haemopoietin receptor superfamily (Parham *et al.*, 2002). IL-23 and IL-12 also share a common subunit, IL-12R β 1, in their receptor complex due to the common p40 subunit. The IL-23R subunit, which binds to the IL-23 p19 subunit, has

an extracellular NH₂-terminal immunoglobulin-like domain and two cytokine receptor domains (Figure 1.7). It is the signalling component of the IL-23 receptor. The IL-12R β 1 subunit contains three membrane-proximal fibronectin type III and two cytokine receptor domains, which interact with IL-23/12 p40 (Langrish *et al.*, 2004; van de Vosse *et al.*, 2003). Although structurally IL-23R belongs to the class I cytokine receptor family, it lacks the three membrane-proximal fibronectin type III-like domains characteristic of the IL-12 receptor subunits (Kastelein *et al.*, 2007).

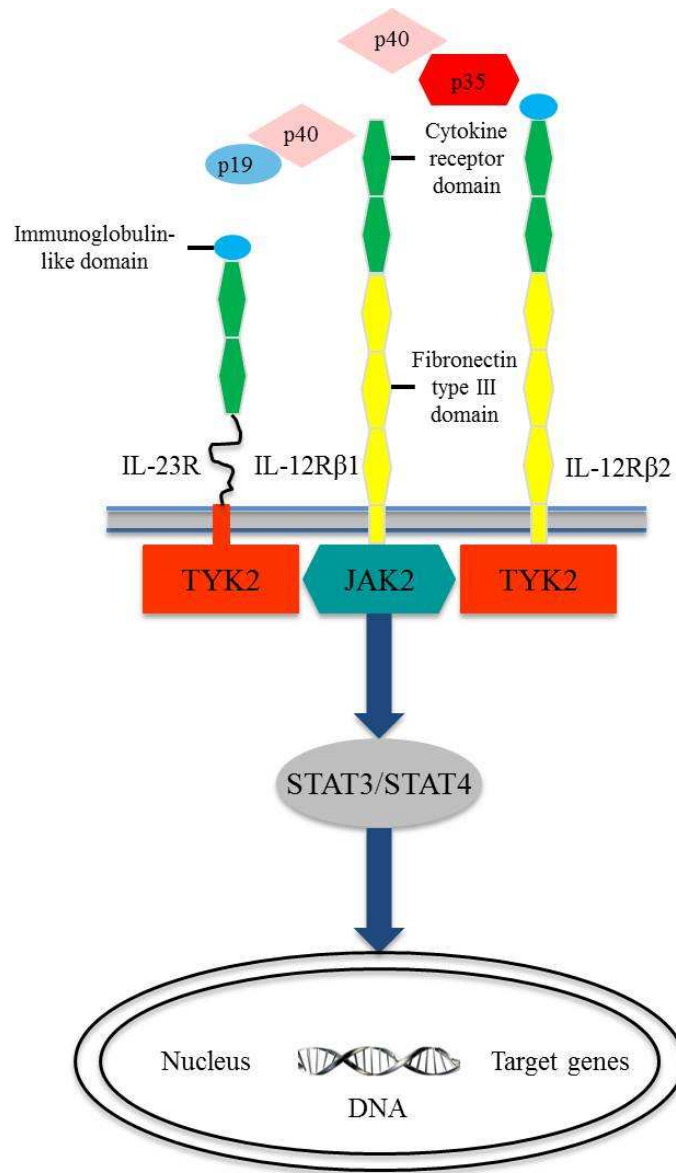


Figure 1.7. The different components of IL-23R, IL-12R and the common STAT3/STAT4 activation pathway. IL-12Rβ1 and IL-12Rβ2 each consists of three fibronectin type III (yellow) domains and two cytokine receptor (green) domains with an additional immunoglobulin-like domain (blue) on the latter. IL-23R closely resembles IL-12Rβ2 but without the fibronectin type III domains. The relative proportions of STAT3 or STAT4 activation dictate whether a cell responds to IL-12 or IL-23. Adapted from Tang *et al.* (2012).

IL-23R is up-regulated on RORγt⁺ Th17 cells in an IL-6-dependent manner (Ivanov *et al.*, 2006) and the generation of normal Th17 cell effector responses requires IL-23R-dependent signalling (McGeachy *et al.*, 2009). IL-23R expression on T cells at the site of inflammation suggests that IL-23R defines a population of infiltrating pro-

inflammatory Th17 cells in the target tissue (Awasthi *et al.*, 2009). In humans, IL-23R is expressed on activated and memory T cells and previously reported to be expressed at low levels on NK cells, monocytes, macrophages and DCs (Parham *et al.*, 2002). However, recently, with the advent of ROR γ t-enhanced green fluorescent protein (eGFP) and IL-23R-eGFP reporter mice (Awasthi *et al.*, 2009), the majority of IL-23R expression has been localised to $\gamma\delta$ T cells and cells of the innate immune system, such as $\gamma\delta$ T cells and ILC3s (Chognard *et al.*, 2014; Zuniga *et al.*, 2013), as discussed in section 1.4.1.

IL-23 induces signalling via IL-23R through the signalling chains JAK2 and tyrosine kinase 2 (Tyk2), leading to activation of STAT3 and STAT4 (Parham *et al.*, 2002). Stimulation of the IL-23R complex induces tyrosine phosphorylation of JAK2 and Tyk2. Consequently, the STATs are phosphorylated, dimerised and translocated into the nucleus, which activates target genes. STAT3 is essential for development of Th17 cells and is required for expression of IL-17A, IL-17F and ROR γ t. The major difference between the signal transduction cascades of IL-23 and IL-12 is the level of STAT3 and STAT4 phosphorylation; IL-23 induces stronger phosphorylation of STAT3 versus STAT4 and the reverse is true for IL-12 (Oppmann *et al.*, 2000).

As mentioned previously, Th17 immune responses and IL-23 play a crucial role in mammalian autoimmune diseases. In humans, polymorphisms in IL-23R have been linked to genetic susceptibility to autoimmune diseases such as psoriasis and IBD (Crohn's disease and ulcerative colitis) (Cargill *et al.*, 2007; Duerr *et al.*, 2006). Development of IL-23R antagonists is a rapidly expanding area of autoimmunity therapy; fifteen IL-23R antagonists are now reported to be in pre-clinical or clinical development from twelve different companies (Tang *et al.*, 2012).

The identification of IL-23 and its receptor in mammals (section 1.3) initiated the discovery of a third Th subset in mammals. Whilst IL-23 is not required for Th17 differentiation, it is vital to the maintenance of Th17 cells in mammals and identifying this cytokine and its receptor in chicken is the first step towards confirming there are Th17 immune responses in birds.

1.5 Infectious disease models

Expression of Th17-related cytokines during infection in the chicken will shed light on the possible involvement of Th17 immune responses in diseases previously recognised as being controlled by Th1 or Th2 immune responses. To this end, archived samples from two Th1 infection models (Marek's disease (MD) and infectious bursal disease) and a Th2 infection model (histomoniasis) were investigated to see if Th17 cytokines were induced. As discussed in section 1.3, the Th17 immune response represents an arm of the adaptive immune system which functions to clear specific types of pathogen not effectively neutralised by Th1 or Th2 immunity (Korn *et al.*, 2009). Current data imply a specialised evolutionary function of Th17 cells to promote protective immunity at mucosal sites against a variety of pathogens (Kolls and Khader, 2010) and in mammals Th17 cells are widely considered to be the main arsenal in host defence against extracellular bacteria (Peck and Mellins, 2010) such as *Staphylococcus aureus*. This is a disease model already established in the chicken at The Roslin Institute and it was used to outline the expression of Th17 cytokines during infection, to further compare avian Th17 immune responses to those in mammals.

1.5.1 Marek's disease

MD is a neuropathic and lymphoproliferative disease of birds caused by MD virus (MDV), which is a highly cell-associated, lymphotropic alphaherpes virus (Schat and Xing, 2000). It is a double-stranded DNA virus with molecular structure and genomic organisation similar to herpes simplex virus. There are three serotypes that differ at both the genome and biological level. Serotype 1 MDV includes all the oncogenic strains and their attenuated forms, serotype 2 MDV are non-oncogenic viruses isolated from chickens and serotype 3 MDV are non-oncogenic viruses isolated from turkeys (Gimeno, 2008). All chicken genotypes are susceptible to MDV infection but they differ greatly in their resistance or susceptibility to clinical disease, e.g. inbred line 6₁ is resistant, whereas line 7₂ is susceptible to clinical disease (Lee *et al.*, 1981). Genes encoded in the chicken MHC locus contribute to MD resistance (Koch *et al.*, 2007). The B21 haplotype is the most resistant and B19 the most susceptible to disease. However, other genes also exert a strong influence on MD resistance. For example, lines 6₁ and 7₂ are MHC-congenic (Cole, 1968) and MD resistance in line 6₁ has been mapped to genetic loci outside the MHC locus (Bumstead, 1998; Smith *et al.*, 2011). MDV vaccines are highly protective, often achieving close to 100% protection under commercial conditions. However, there is growing evidence that virulence of MDV is increasing due to the intensive use of vaccines (Witter, 1997).

MDV is not vertically transmitted through infected eggs; however, chicks become infected after hatching via a heavily contaminated environment. The natural route of MDV infection is via the respiratory tract following inhalation of infectious cell-free MDV present in dander shed from feather follicles. The virus is incredibly stable in

the environment. Various factors such as virus strain and dosage, gender and genetic resistance of the host, presence of maternal antibodies and environmental factors affect the clinical outcome (Nair, 2014).

Based on the 'Cornell Model' (Calnek, 1986), there are four phases of MDV pathogenesis (Figure 1.8). MDV is inhaled and infects macrophages in the lung. During the early cytolytic phase, B cells become infected as a result of infected macrophages tracking to the spleen. T cells are activated by infected B cells and subsequently are also infected with MDV. At this stage, the virus is able to enter a latent phase, where depending on the pathotype of the virus and the genotype of the host, T cells are transformed and proliferate to form malignant tumours. MDV is shed into the environment in the feather follicle epithelium.

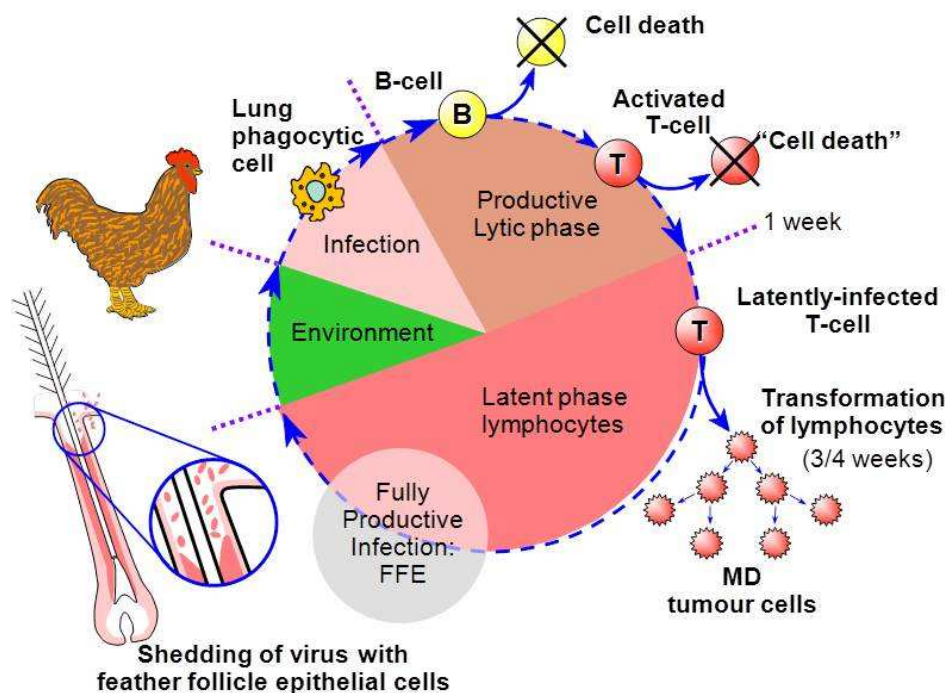


Figure 1.8. The infection cycle of MDV. The virus enters the chicken and infects phagocytes. B cells and T cells are subsequently infected and some T cells enter a state of latency. The T cells can then transform and cause tumours in the bird. The virus is shed within the feather follicle epithelial cells (Davison and Nair, 2008).

The immune response to MDV infection involves CTL responses and antibody production. The defining role of cytokines in MDV pathogenesis and immunity remains unclear (Heidari *et al.*, 2008), it has been established that IFN- γ expression is up-regulated during MDV infection and a predominantly Th1-type immune response is seen in chickens infected with MDV (Volpini *et al.*, 1995; Xing and Schat, 2000). Our group also showed increased IFN- γ mRNA levels in both susceptible and resistant genotypes infected with MDV. Further, increased mRNA expression of IL-6 and IL-18 was detected in MDV infected susceptible genotypes compared to resistant genotypes (Kaiser *et al.*, 2003). Th17 cytokines have yet to be measured during MD.

1.5.2 Infectious bursal disease

Infectious bursal disease virus (IBDV) is a non-enveloped double-stranded RNA virus of the family *Birnaviridae*. Its simple structure makes the virus highly resistant in the environment and this is an important issue in the control of the disease. There are two serotypes recognised: serotype 1, which only causes disease in chickens and serotype 2, which was isolated from turkeys and is considered non-pathogenic in chickens (McFerran *et al.*, 1980). Based on virulence, serotype 1 strains are classified as classical, intermediate, virulent and very virulent (van den Berg *et al.*, 2000).

IBDV causes immunosuppression in chickens as a result of aggressive destruction of B cells in the lymphoid organs, particularly the bursa of Fabricius. In the acute form, birds show signs of depression and dehydration. *In vitro* studies have shown that IBDV replicates in proliferating B cells (Muller, 1986) and the severity of the disease

is directly related to the number of susceptible B cells present in the bursa of Fabricius. Consequently, chicks between 3-6 weeks of age are most susceptible to disease (Ingrao *et al.*, 2013). Bursectomised chickens survive infection with IBDV (Okoye and Uzoukwu, 1990).

It is hypothesised that IBDV enters the host via the oral route and gut-associated macrophages transport the virus from the digestive tract to the bursa of Fabricius and other peripheral organs (Kaufer and Weiss, 1976). All parts of the chicken immune system are affected during infection with IBDV (Figure 1.9). The virus destroys B cells, attracts T cells and activates macrophages but the molecular basis for IBDV pathogenicity is still poorly understood (Ingrao *et al.*, 2013). What is known is that bursal T cells are activated and gene transcription of pro-inflammatory cytokines, e.g. chIL-1 β , chIL-6, CXCL2 and chIFN- γ , is up-regulated during the acute phase of IBDV infection (Eldaghayes *et al.*, 2006). Further, Liu *et al.* (2010) demonstrated up-regulation of Th1 and Th2 cytokines during IBDV infection, indicating that the primary immune response is likely to be cell-mediated. Th17 cytokines have yet to be measured in Infectious bursal disease.

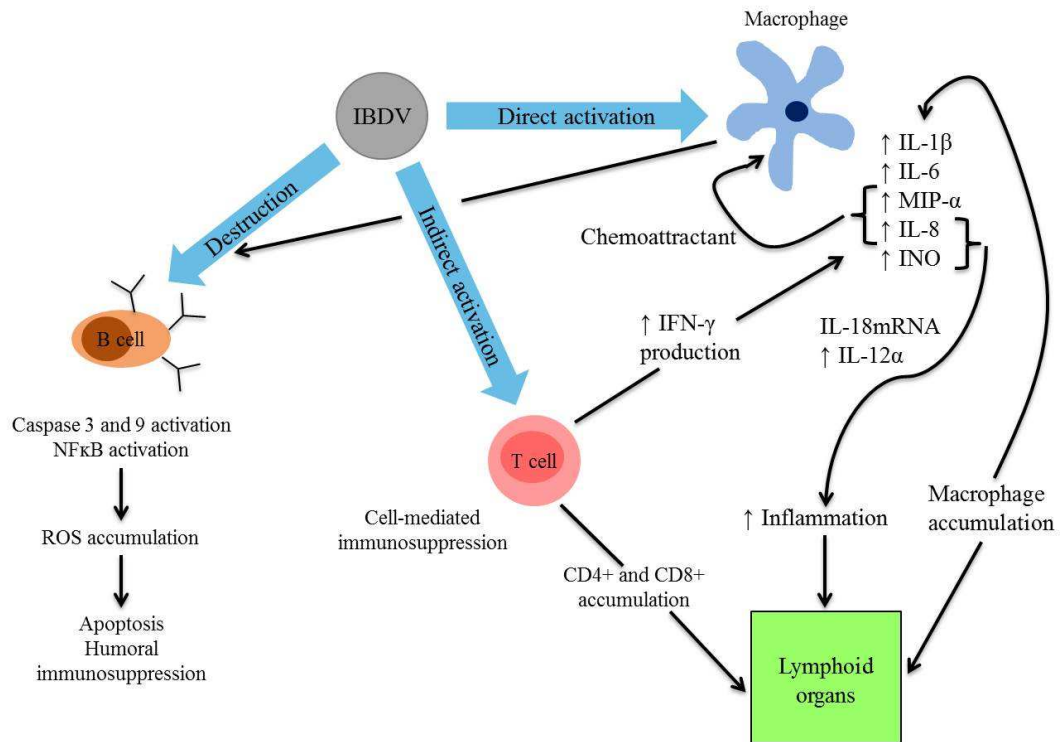


Figure 1.9. Interactions between IBDV and the host immune cells (Ingrao *et al.*, 2013).

1.5.3 Histomoniasis

The trichomonad *Histomonas meleagridis* (*H. meleagridis*) is a flagellate, amoeboid, extracellular protozoan parasite that causes the disease histomoniasis in galliform birds (formerly known as enterohepatitis or ‘blackhead’ disease). Typically, birds show signs of anorexia and depression due to enterohepatitis. It can infect all species of galliform birds but its pathogenicity varies markedly between species. The most prominent lesions are seen in the caeca. Chickens recover quickly but become healthy carriers of the protozoan whereas turkeys show more severe caecal lesions as well as liver lesions and mortality can be very high, approaching nearly 100%. Birds become infected with *H. meleagridis* by ingesting infected embryonated eggs of *Heterakis gallinarum* (*H. gallinarum*), which is a caecal worm.

The importance of the caecal worm as a *H. meleagridis* vector was discovered by Graybill (1921) and the chicken was identified as the best host for caecal worms and a reservoir of infection (Lund and Chute, 1974). In turkeys, bird-to-bird transmission of *H. meleagridis* is possible by ‘cloacal drinking’, where protozoa shed in the faeces are taken up by the cloaca and transported by retrograde peristalsis to the caeca (Hu and McDougald, 2003). The ‘cloacal drinking’ phenomena is also well-known in chickens but bird-to-bird transmission of *H. meleagridis* infection has not been shown (Hu *et al.*, 2006). Under experimental conditions, intra-cloacal infection with infected caeca and liver tissues eliminates the need for the vector thus avoiding any immune response to *H. gallinarum* complicating the immune response to *H. meleagridis* (Powell *et al.*, 2009). This is important when investigating cytokine expression in response to *H. meleagridis* infection so that any immune response measured can be more confidently attributed to this infection and not to the vector *H. gallinarum* as well.

The chicken mounts a typical Th2-type immune response to *H. meleagridis* infection, with increased expression of IL-13 (Powell *et al.*, 2009). Further, Powell *et al.* (2009) also showed the chicken mounts an earlier innate immune response in the caecal tonsil compared to the turkey when infected with *H. meleagridis*, possibly limiting the movement of protozoa from the caeca to the liver in the chicken. The expression of Th17 cytokines in *H. meleagridis* infection in chicken has not been investigated to-date.

1.5.4 *Staphylococcus aureus* infection

Staphylococcus aureus (*S. aureus*) is a Gram-positive, catalase-positive, coccoid bacteria. It is a commensal bacteria of the skin and mucosal surfaces and an opportunistic pathogen. *S. aureus* is associated with several infections of poultry including septic arthritis, subdermal abscesses ('bumble foot') and gangrenous dermatitis. *S. aureus* is a ubiquitous organism in the poultry house environment and can be isolated from the litter, dust and feathers (Skeeles, 1997). Bacterial chondronecrosis with osteomyelitis (BCO) was first reported as a cause of lameness in commercial broiler chickens in 1972 (Nairn and Watson). It is now recognised as the most common cause of lameness in commercial birds (Dinev, 2009; McNamee, 1999; McNamee and Smyth, 2000) with *S. aureus* being the most common cause of BCO in commercial chickens (McNamee and Smyth, 2000).

The exact pathogenesis of BCO remains unclear. *S. aureus* must enter the circulatory system in order for bacteraemia to develop. Injury, an open naval post-hatching and the respiratory system have all been implicated as possible routes of entry into the bloodstream (reviewed by McNamee and Smyth (2000)). Once in the circulation, staphylococci have a high affinity for collagen-rich surfaces (Nade and Speers, 1987) and tend to localise in the growth plate of actively growing bones (Speers and Nade, 1985), explaining the higher incidence of BCO in young chickens. Since bacteraemia is required for BCO development, development of the disease has been studied by injecting *S. aureus* intravenously. An established experimental BCO model, this has also been used extensively as a model for acute haematogenous staphylococcal osteomyelitis of human infants (Daum *et al.*, 1990; Emslie *et al.*, 1983).

The strong relationship of Th17 cells with the mammalian immune response to *S. aureus* infection is well established (Cho *et al.*, 2010; Frodermann *et al.*, 2011; Henningsson *et al.*, 2010). Early innate signalling, which drives the appropriate adaptive response to a pathogen, in response to *S. aureus* infection, results in production of IL-23 (Volz *et al.*, 2010). Also, humans and mice lacking Th17 immune responses show increased susceptibility to *S. aureus* infections (Ma *et al.*, 2008; Renner *et al.*, 2008). Of the pathogens in mammals known to drive Th17 immune responses and, therefore, require a Th17 adaptive response to overcome infection, *S. aureus* infection is currently the only disease model established in poultry.

The theory is the disease models, MDV and IBDV, typically inducing Th1-type immune responses in the chicken, the Th2-inducing infection, *H. meleagridis* in chicken and the mammalian Th17-inducing immune responses of *S. aureus*, would provide a fundamental basis to further investigations of Th17 immune responses in birds.

1.6 Hypothesis and aims of the project

As previously discussed, there are both similarities and significant differences between the avian and mammalian immune system. The evolutionary distance between birds and mammals, and the speed of orthologous sequence divergence under selective pressure from pathogens, has resulted in relatively slow progress in cloning and characterising avian cytokines. Mammalian reagents and bioassays often do not cross-react with avian molecules as aa sequence identities are often as low as 25% (Kaiser *et al.*, 2005). This project aims to establish whether Th17 and associated immune responses exist in the chicken. The hypothesis was that chickens have Th17 cells, are able to mount Th17-associated immune responses and that the driving cytokine IL-23 has a similar biological role to that seen in mammals.

To test the hypothesis, the aim will be to clone and characterise IL-17F, IL-23, IL-23R and ROR γ t, and to investigate the expression and effects of chicken Th17 cytokines *in vitro* on chicken-derived tissues and during infection in the chicken. The biological activity and effects of IL-23 on immune cells in the chicken will also be investigated. Additionally, the biological activity and effects of IL-23 on immune cells in the chicken will be investigated. Finally, attempts will be made to drive CD4⁺ T cells to a Th17 phenotype, using the chicken orthologues of the cytokines that drive such differentiation in mammals.

Chapter 2 Materials and methods

2.1 *In silico* software packages

2.1.1 *ClustalX*

ClustalX allows multiple alignments of nucleotide (nt) or aa sequences (Thompson *et al.*, 1994). ClustalX is currently maintained at the Conway Institute, University College Dublin, Dublin.

2.1.2 *Multalin*

Multalin allows multiple alignments of nt or aa sequences (Corpet, 1988).

2.1.3 *GeneDoc*

GeneDoc is a multiple sequence alignment software tool, which allows editing, analysing and a shading utility for use in Windows (Nicholas *et al.*, 1997). GeneDoc was used to produce highly configurable exported figures of ClustalX aa alignments. Further information on the software can be found at:
<http://www.psc.edu/biomed/genedoc>

2.1.4 *SignalP3.0*

SignalP3.0 (Centre for Biological Sequence Analysis, Technical University of Denmark DTU; <http://www.cbs.dtu.dk/services/SignalP/>) is an online server, which predicts the existence and position of signal peptides as well as the exact residues where cleavage will occur in an aa sequence (Bendtsen *et al.*, 2004).

2.2 Sources of chicken tissues and cells

2.2.1 *J line birds*

All tissue samples were extracted from vaccinated 4-9-week-old J line birds, from a flock maintained in The Roslin Institute Poultry Unit. All birds were vaccinated against MDV, coccidiosis, Newcastle Disease virus, infectious bronchitis virus, IBDV, chicken anaemia virus, avian encephalomyelitis virus and infectious laryngotracheitis virus.

2.2.2 *MDV challenged line 6₁ and line 7₂ birds*

Two-week-old White Leghorn specific pathogen free (SPF) chickens of the inbred lines 6₁ (MDV resistant) and 7₂ (MDV susceptible) were challenged with MDV strain RB1B as described by Smith *et al.* (2011). Archived RNA samples from spleen at 2, 3 and 4 days post-infection (dpi) from 2 uninfected control birds and 3 infected birds were used.

2.2.3 *IBDV challenged birds*

Four-week-old out-bred Rhode Island Red chickens reared from the SPF flock maintained at the Institute of Animal Health, Compton, United Kingdom were challenged with a very virulent strain of IBDV as described by Maghoub (2010). Archived RNA samples from caecal tonsil at 1, 3 and 4 dpi from 1 uninfected control bird and 3 infected birds were used.

2.2.4 *Histomonas meleagridis* challenged birds

Breeder cockerels at 4-weeks of age were challenged with *H. meleagridis* as described in the second chicken study by Powell *et al.* (2009). Archived RNA

samples from caecal tonsil at 1, 2, 3, 4, 6, 8, 10, 12, 18, 24 and 30 dpi from 2 uninfected control birds and 3 infected birds were used.

2.2.5 *Staphylococcus aureus* challenged birds (pilot study)

This study was carried out in collaboration with Prof. Ross Fitzgerald and Dr Andreas Lengeling. Four-week-old female Cobb-Vantress broilers, obtained as eggs from Cobb-Vantress, were hatched and reared in The Roslin Poultry Unit, University of Edinburgh, United Kingdom, before being transferred to the high security unit, Moredun Research Institute, Penicuik, United Kingdom. Bacterial virulence of the wild-type *S. aureus* CIX2 strain was compared to an isogenic mutant strain of *S. aureus* in which a β -converting phage (ϕ Av β) had been deleted from the bacterial genome. Twenty-five 29 day-old female Cobb broilers were divided into 4 groups: wild-type (7 birds), mutant (7 birds), co-infected (6 birds) and control (5 birds). Each bird was infected intravenously with the same volume (200 μ l) and number of bacteria (4×10^4 colony forming units (CFU)/ml) of either the wild-type or mutant *S. aureus* strains, or co-infected with equal numbers of both bacterial strains in the same volume of inocula (total 8×10^4 CFU/ml). The control group was administered phosphate buffered saline (PBS) (Central Services Unit, The Roslin Institute) intravenously. The birds were housed in pens on the floor in the same room and monitored for disease phenotypes over a period of 11 days. Half of the birds were culled and sampled 6 dpi and the remaining birds were culled and sampled 11 dpi. Tissues sampled (spleen, bursa, thymus, liver and bone marrow from the femur) were placed in RNeasy lysis buffer for RNA extraction (section 2.3.2) and Th17 cytokine mRNA expression by TaqMan® RT-qPCR assay as described in sections 1.1.1.1 and 2.4.3.6.

2.2.6 *Chicken tissue panel*

Tissues (brain, muscle, heart, liver, kidney, lung, skin, crop, gizzard, upper gut, caecum, mid-gut, caecal tonsil, Meckel's diverticulum, Harderian gland, bursa of Fabricius, femurs for bone marrow, spleen and thymus) were harvested from J line birds at 4-9 weeks of age. Tissues were collected and stored in *RNAlater* (Ambion®) at -20°C until RNA was purified.

2.3 Plasmid purification

2.3.1 *DNA isolation*

2.3.1.1 *Minipreps (small scale)*

Bacterial colonies were picked from LB plates and inoculated into 5 ml of LB containing 100 µg/ml of ampicillin. These were cultured overnight at 37°C with shaking at 200 rpm. These cells were pelleted with centrifugation at 1800 x g for 15 min at 4°C and the supernatant discarded. Minipreps were carried out using the QIAprep Spin Miniprep kit (Qiagen) following the manufacturer's protocol. DNA concentration was determined using a Nanodrop ND 1000 spectrophotometer.

2.3.1.2 *Maxipreps (large scale)*

Bacterial colonies were picked from LB plates and inoculated into 5 ml of LB broth containing 100 µg/ml of ampicillin. These were cultured for 6 h at 37°C with shaking at 200 rpm. Day culture (2.5 ml) were used to inoculate 200 ml of LB broth containing 100 µg/ml of ampicillin in a 1 l flask and cultured overnight at 37°C with shaking at 200 rpm. These cells were pelleted with centrifugation at 6000 x g for 15 min at 4°C and the supernatant discarded. Maxipreps were carried out using an

Endofree Plasmid Maxi kit (Qiagen) following the manufacturer's protocol. DNA concentration was determined using a Nanodrop ND 1000 spectrophotometer.

2.3.2 RNA isolation

2.3.2.1 Isolation of total RNA from chicken tissue

Total RNA was purified from chicken tissue using the RNeasy® Mini Kit (Qiagen) following the manufacturers' protocol for purification of total RNA from animal tissues. Fresh tissues were stabilised in RNAlater® (Ambion®) and frozen at -20°C until processed. After thawing, 30 mg of tissue was lysed and homogenised using a rotor-stator homogeniser in 600 µl of buffer RLT with β-mercaptoethanol (1/100) in a lysing matrix tube (MP Biomedicals). All samples were homogenised for 40 s. Following homogenisation, samples were centrifuged at 13,000 x g for 3 min. The supernatant was pipetted off and 1 volume of 70% ethanol (RNase-free) was added. Samples were applied to RNeasy® mini columns and centrifuged at 8,000 x g for 15 s. Residual fluids were discarded before the addition of 700 µl of buffer RW1 and centrifugation again at 8,000 x g for 15 s. Residual fluids were discarded and 500 µl of buffer RPE was added, followed by centrifugation at 8,000 x g for 15 s. This step was repeated a second time before the RNeasy® mini column was placed in a new collection tube and centrifuged at 8,000 x g for 1 min. RNA was eluted into a clean 1.5 ml tube with 50 µl nuclease-treated H₂O (Ambion®) by centrifugation at 8,000 x g for 1 min. RNA was stored at -20°C prior to use.

2.3.2.2 DNase treatment of RNA samples

DNase digestion is generally not required with RNeasy® Kits since RNeasy® silica membrane technology efficiently removes most of the DNA without DNase

treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA. DNA can also be removed by DNase digestion following RNA isolation.

2.3.2.2.1 On-column DNase treatment during RNA extraction

RNA samples from the *S. aureus* pilot study were on-column DNase treated during RNA extraction. An RNase-Free DNase Set (Qiagen) was used according to the manufacturer's protocol to digest DNA on-column during RNA purification as in section 2.3.2.1. Briefly, the sample was applied to an RNeasy® mini column, centrifuged at 8,000 x *g* for 15 s and the residual fluid discarded. Buffer RW1 (350 µl) were applied to the RNeasy® mini column and centrifuged again at 8,000 x *g* for 15 s. The residual fluid was discarded. DNase I stock solution (10 µl) was added to 70 µl buffer RDD and mixed gently by inversion. DNase I incubation mix (80 µl) was added directly to the RNeasy® mini column and incubated at RT for 15 min. Buffer RW1 (350 µl) was added to the RNeasy® mini column and centrifuged at 8,000 x *g* for 15 s. The residual fluid was discarded. The protocol was continued as described in section 2.3.2.1 with the addition of 500 µl buffer RPE.

2.3.2.2.2 Removal of contaminating DNA from RNA preparations

To compare mRNA detection by TaqMan® in DNase-treated versus non-DNase-treated RNA samples, a TURBO DNA-free™ kit (Ambion®) was used according to the manufacturers' protocol. In brief, 0.1 volumes of 10X TURBO DNase buffer and 1 µl TURBO DNase were added to 20 µl RNA and incubated at 37°C for 30 min. Two microlitres re-suspended DNase inactivation reagent were added, mixed well and incubated at RT for 5 min, mixing occasionally. The sample was centrifuged at

10,000 x *g* for 1.5 min to pellet DNA and DNase enzymes. The RNA in the supernatant was carefully pipetted off and transferred to a clean Eppendorf tube for storage at -20°C prior to use.

2.3.3 Agarose gel electrophoresis of DNA

Products (5 µl) from all PCR and restriction enzyme digests were analysed by gel electrophoresis. The products were combined with 6X loading buffer (Invitrogen) and loaded into a 0.7% (products larger than 500 nt) or 1.5% (products smaller than 500 nt) gel, composed of agarose dissolved in Tris-acetate-ethylenediaminetetraacetic acid (EDTA) (TAE) buffer. 1X SYBR® Safe (Life Technologies) was incorporated into the gel to allow DNA to be visualised under an ultraviolet illuminator. Five microlitres of DNA marker ladder (Invitrogen TrackIt 100 bp ladder or Thermo Scientific GeneRuler™ DNA Ladder Mix) was used to estimate product size.

2.3.4 PCR purification

Single PCR products of interest were purified using a QIAquick PCR Purification kit (Qiagen) following the manufacturer's protocol. Five volumes of buffer PB were added to 1 volume PCR reaction, mixed, and added to a QIAquick spin column in a 2 ml collection tube. Centrifugation was carried out at 13,000 x *g* for 1 min. The flow-through was discarded and the column washed with 750 µl buffer PE. Residual fluids were discarded following centrifugation at 13,000 x *g* for 1 min. A further centrifugation at 13,000 x *g* for 1 min eliminated possible carryover of ethanol. Elution was carried out in a clean 1.5 ml tube with 30 µl of buffer EB by centrifugation at 13,000 x *g* for 1 min following 1 min incubation at RT.

2.3.5 Gel purification

Where multiple products of interest were present in one reaction, the cDNA was electrophoresed on an agarose gel. The fragments of interest were excised with a clean scalpel and purified using a QIAquick Gel Extraction kit (Qiagen), following the manufacturers' protocol. Briefly, buffer QG was added at 3 times the volume of the gel and incubated at 50°C. One gel volume of isopropanol was added to the sample, mixed and added to a QIAquick spin column in a 2 ml collection tube. The tube was centrifuged at 13,000 x *g* for 1 min. Residual fluid was discarded, the column washed with 500 µl of buffer QG and centrifuged at 13,000 x *g* for 1 min. Residual fluid was discarded, the column washed with 750 µl buffer PE and centrifuged at 13,000 x *g* for 1 min. Contaminants were discarded in the flow-through and the column centrifuged for a further 1 min at 13,000 x *g*. Elution was carried out in a clean tube with 30 µl of buffer EB and centrifuged at 13,000 x *g* for 1 min following 1 min incubation at RT.

2.4 DNA and RNA amplification

2.4.1 Oligonucleotide primer design

Oligonucleotide primers were used to generate cDNA templates, amplify cDNA in PCR and RACE-PCR, measure expression of mRNA by TaqMan® and SBYR® Green assays and for sequencing of PCR products (Table 2.1, Table 2.2, Table 2.3, Table 2.4 and Table 2.5). Primers and probes were designed manually with the exception of TaqMan® primers and probe sets, which were designed using Primer Express v3 (Applied Biosystems). IL-23 p19 TaqMan® primers and probe were custom-designed by Applied Biosystems.

Table 2.1. Primer sequences for PCR

Primer name	Target for amplification	Sequence (5' – 3')
OligodT_20	mRNA transcripts	TTTTTTTTTTTTTTTTTTTT
IL-17A_F1	IL-17A cDNA	ATGTCTCCGATCCCTTGTTT
IL-17A_R1	IL-17A cDNA	TTAAGCCTGGTGCTGGATCA
IL-17F_F3	IL-17F cDNA	ATGGCTTTTGCTAGCTGTGCTGC
IL-17F_R2	IL-17F cDNA	CTAGGACTGGTGCTGGATGACC
IL-23_F1	IL-23 EST cDNA	GCACGAGGAGGACATGGA
IL-23_R1	IL-23 EST cDNA	GGAACGCATGGGGACCTG
IL-23_F2	IL-23 p19 cDNA	ATGGCCCCGCTCCGCCGC
IL-23_R3	IL-23 p19 cDNA	TCAGCGGGTGCTGGCGCC
IL-23R_F10	IL-23R cDNA	ATGAACGCTTTCACTGTGCTTGAA
IL-23R_R1b	IL-23R cDNA	TCAAAACAATCTTCCGATGCTCTG

Table 2.2. Primer sequences for 5' and 3' RACE-PCR

Primer name	Sequence (5' – 3')
IL-23_GSP_F1	GACATCTTCGCCACACACCCACA
IL-23_GSP_RC1	CAGAGCGTGCTGTTTCGTGTCCAGC
IL-23 GSP NF1	GCTGAAGGCGGTGCTGGAGGAGC
IL-23 GSP NRC1	TCGCAGGCGTCGCTGCAGCGGATG
IL-23R GSP F1	GCACCTCCCCATAACACGGCCAC
IL-23R GSP RC1	GCTGTTTGTGAATCACTCTTCTCC
IL-23R GSP NF1	GAGGACGTCAGTGACTACAAACCC
IL-23R GSP NRC1	GGAAGGACTTTATCACCTTGCTG

Table 2.3. Primer sequences for SYBR® Green.

Primer Name	Sequence (5' – 3')
p19_F1	GTGGAGAGGAGGACATGGAG
p19_F2	CTGGGGACAATGAAGGAGTC
p19_F3	GGGACAATGAAGGAGTCGCA
p19_F4	ATGGAGGGGGAGTG GCC
p19_F5	GAGCGGGGTCCGTC
p19_F6	GTCCGTCTGGGTGGAG
p19_R1	GGTGTGTGGCGAAGATGTC
p19_R2	AGAGCGTGCTGTTCGTGT C
p19_R3	GGCACACTCCCCCTCCAT
p19_R4	GTGTGGCGAAGATGTCGG AG
p19_R5	CGAGGCTCAGCAGCTC
p19_R6	CGAGTGTGCCGTGCTG

Table 2.4. Primer and probe sequences for TaqMan®. a indicates MGB probe. F = forward primer; R = reverse primer; P = probe; ex-COS = RNA for standards expressed in COS-7 cells.

Target	Sequence (5' – 3')	Standard	Primer conc. (μM)
28S	F: GGCGAAGCCAGAGGAAACT R: GACGACCGATTTGCACGTC P: AGGACCGCTACGGACCTCCACCA	LPS stimulated HD11	0.6
IL-1β	F: GCTCTACATGTCGTGTGTGATGAG R: TGTCGATGTCCCGCATGA P: CCACACTGCAGCTGGAGGAAGCC	IL-1β ex-COS	0.4
IL-2	F: TTGGAAAATATCAAGAACAAGATTCATC R: TCCCAGGTAACACTGCAGAGTTT P: ACTGAGACCCAGGAGTGCACCCAGC	IL-2 ex-COS	0.6
IL-6	F: GCTCGCCGGCTTCGA R: GGTAGGTCTGAAAGGCGAACAG P: AGGAGAAATGCCTGACGAAGCTCTCCA	IL-6 ex-COS	0.2
IL-12α	F: TGGCCGCTGCAAACG R: ACCTCTTCAAGGGTGCACCTCA P: CCAGCGTCCTCTGCTTCTGCACCTT	IL-12α ex-COS	0.4
IL-17A	F: CATGGGATTACAGGATCGATGA R: GCGGCACTGGGCATCA P: ACAACCGCTTCCCCCGCTTGG	IL-17A ex-COS	0.4
IL-17F	F: TGACCCTGCCTCTAGGATGATC R: GGGTCCTCATCGAGCCTGTA P: CAGGAATCGGTCTCTCGTCTCTTGG	IL-17F ex-COS	0.6
IL-21	F: AAAAGATGTGGTGAAAGATAAGGATGT R: GCTGTGAGCAGGCATCCA P: TGCTGCATACACCAGAAAACCTGGG	IL-21 ex-COS	0.2
IL-22	F: TCAACTTCCAGCAGCCCTACAT R: TGATCTGAGAGCCTGGCCATT P: AGGAATCGCACCTACACCTTGGCTGA	IL-22 ex-COS	0.8
IL-23 p19	F: GGGCTGCAGCACTACCA R: GTGGGTGTGTGGCGAAGAT P ^a : CAGGCTGGGCTCCGAC Copyright © 2014 Life Technologies Corporation. Used under permission.	IL-23flexi ex-COS	-
IL-23R	F: ACAGCCATGAGGAAGAGTTCTTTT R: CATACACCGACACGTTGATGTG P: AGGACAGCCACACCTACATCCAAGAGGAC	IL-23R ex-COS	0.4
IFNγ	F: GTGAAGAAGGTGAAAGATATCATGGA R: GCTTTGCGCTGGATTCTCA P: TGGCCAAGCTCCCGATGAACGA	IFNγ ex-COS	0.6

Table 2.5. Primer sequences for sequencing vector inserts.

Primer Name	Sequence (5' – 3')
T7	TAATACGACTCACTATAGG
SP6	ATTTAGGTGACACTATAG
pcDNA3.1 reverse	TAGAAGGCACAGTCGAGG
pTarget_Rev	TTACGCCAAGTTATTTAGGTGACA
IL-23R_F9	TGGCATGTGACAGCATGGGAC

2.4.2 *cDNA synthesis*

Reverse transcription was carried out with 5 µl total RNA as a template in a standard 20 µl reaction, which contained 5X First Strand buffer (Invitrogen) (4 µl) mixed with 1 µl of 0.1 M dithiothreitol (DTT), 1 µl PCR Grade Nucleotide Mix (Roche), 1 µl of oligo(dT)20 (50 µM) or 2 pmol gene-specific reverse primer (Table 2.6) 1 µl RNAsin (Promega), 1 µl SuperScript III (Invitrogen) and 6 µl nuclease-treated H₂O (Ambion®). First strand synthesis was carried out for 1 h at 50°C if using oligo(dT)20 primer or 1 h at 55°C if using a gene-specific reverse primer, followed by incubation at 70°C for 15 min to inactivate the reaction. The cDNA products were used as a template to carry out PCR. If the cDNA product was to be used for SYBR® Green quantitative RT-PCR (section 2.4.3.8) then complementary RNA was removed by the addition of 1 µl RNase H (Life Technologies) and further incubation at 37°C for 20 min.

Table 2.6. Primers used for reverse transcription.

Gene for amplification	Reverse primer used for reverse transcription
IL-17F	IL-17F_R2
IL-23	IL-23_R3
IL-23R	IL-23R_R1b

2.4.3 *PCR amplification of DNA*

2.4.3.1 *Standard PCR*

PCR reactions were performed in a standard 30 µl reaction containing 1 µl of cDNA template, 1X final volume of PCR buffer (Promega), 1.5 mM magnesium chloride (MgCl₂) (Promega), 0.4 mM PCR Grade Nucleotide Mix, 12 µM of each primer (Table 2.1) and 0.5 units of Go Taq® DNA polymerase (Promega). Thermal cycling was performed as described in Table 2.7.

Table 2.7. Cycling conditions for standard PCR.

Step	IL-17F	Cycles
Initial denaturation	95°C, 3 min	1 x
Denaturation	95°C, 1 min	30 x
Annealing	65°C, 2 min	
Extension	72°C, 2 min	
Final extension	72°C, 10 min	1 x
Cooling	12°C, hold	

2.4.3.2 IL-23 PCR

The Expand High Fidelity PCR system (Roche) was used to clone chIL-23 p19. A 30 µl reaction contained 0.6 µl of cDNA template, 3 µl 10X Expand High Fidelity buffer, 0.2 mM dNTPs, final concentration 0.3 µM of each primer (Table 2.1) and 1.6 U of Expand High Fidelity enzyme mix. Thermal cycling was performed as described in Table 2.8.

Table 2.8. Cycling conditions for Expand High Fidelity PCR System (Roche).

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	2 min	1 x
Denaturation	95	40 s	8 x
Annealing	64	40 s	
Extension	72	1 min	
Denaturation	95	40 s	8 x
Annealing	63	40 s	
Extension	72	1 min	
Denaturation	95	40 s	25 x
Annealing	62	40 s	
Extension	72	1 min	
Final extension	72	10 min	1 x
Cooling	12	Hold	

2.4.3.3 IL-23R PCR

The GC-Rich PCR System, dNTPack (Roche) was used to clone chIL-23R. A 30 µl reaction contained 1 µl of cDNA template, 6 µl 5X GC-RICH PCR reaction buffer, 1 µl PCR Grade Nucleotide Mix, final reaction concentration 0.2 µM of each primer (Table 2.1), 4 mM of MgCl₂, and 0.5 M Resolution Solution and 1.25 U GC-RICH PCR system enzyme mix. Thermal cycling was performed as described in Table 2.9.

Table 2.9. Cycling conditions for GC-Rich PCR System, dNTPack (Roche).

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	3 min	1 x
Denaturation	95	30 s	8 x
Annealing	62	30 s	
Extension	72	2.5 min	
Denaturation	94	30 s	8 x
Annealing	62	30 s	
Extension	72	3 min + 5 s cycle elongation for each successive cycle	
Final extension	72	7 min	1 x
Cooling	12	Hold	

2.4.3.4 5' and 3' Rapid amplification of cDNA ends (RACE)

5' and 3' RACE were performed using a SMARTer™ RACE cDNA amplification kit (Clontech) and an Advantage PCR 2 kit (Clontech). The manufacturer's instructions were followed and primers (Table 2.2) were designed according to the instructions (a melting temperature (T_m) of more than 70°C). RNA from stimulated splenocytes, thymocytes and DCs were used as templates for cDNA. Thermal cycling conditions were performed as in Table 2.10.

Table 2.10. Cycling conditions for 5' and 3' RACE-PCR.

Step	Temperature (°C)	Time	Cycles
Denaturation	94	30 s	5 x
Extension	72	3 min	
Denaturation	94	30 s	5 x
Annealing	70	30 s	
Extension	72	3 min	
Denaturation	94	30 s	25 x
Annealing	68	30 s	
Extension	72	3 min	
Final extension	72	8 min	1 x
Cooling	12	Hold	

2.4.3.5 Real-time quantitative RT-PCR (TaqMan®)

A master mix was prepared using 5 µl Fast Universal PCR master mix (2X) (Applied Biosystems), 0.125 µl MultiScribe™ and RNase inhibitor, 2.5 µl RNA (diluted 1/5), 0.5 µl of the optimal primer dilution and 0.25 µl (5 µM) of probe (Table 2.4) and made up to 10 µl with nuclease-treated H₂O (Ambion®).

COS-7 cell RNA (for transfection and RNA isolation see sections 2.8.3 and 2.3.2.1 respectively) containing the gene of interest was used to set up a standard curve for the TaqMan® assays, using 10-fold serial dilutions. The test RNA was diluted 1/5 for cytokine mRNA expression analysis.

28S ribosomal RNA (rRNA) expression was used as a reference gene for standardisation of mRNA levels. The RNA samples were assayed at a dilution of 1/500. A standard curve was also set up for this assay using stimulated HD11 RNA (kindly supplied by Lisa Rothwell) (HD11 cells stimulated with 200 ng/ml lipopolysaccharide (LPS) (Sigma) for 6 h) at a starting dilution of 1/10.

MicroAmp FAST 96-well plates were loaded with 7.5 µl master mix per well. 2.5 µl of nuclease-treated water were loaded into no template control (NTC) wells and 2.5 µl of RNA loaded in triplicate. An optical adhesive cover was then applied and the plate briefly centrifuged. The plate was then placed on the ABI 7500 FAST sequence detection system (Applied Biosystems). The amplification conditions used are outlined in Table 2.11. Data were collected during the 60°C step.

Table 2.11. Cycling conditions for TaqMan®.

Step	Temperature (°C)	Time	Cycles
RT	48	30 min	1 x
Denaturation	95	20 s	1 x
Denaturation	95	3 s	40 x
Annealing/extension	60	30 s	

2.4.3.6 IL-23 custom TaqMan® assay

A master mix was prepared using 5 µl Fast Universal PCR master mix (2X) (Applied Biosystems), 0.125 µl MultiScribe™ and RNase inhibitor, 2.5 µl RNA (diluted 1/5), 0.5 µl of IL-23 Custom TaqMan Assay Mix (20X) (Table 2.4) and 1.75 µl nuclease-treated H₂O.

For the standard curve, reference gene and cycling conditions used see section 1.1.1.1.

2.4.3.7 Analysis of TaqMan®

The data output were expressed as cycle threshold (Ct), the cycle at which the fluorescence from the reporter dye passed a set threshold. The threshold was appropriately set for each target gene by the 7500 Software v2.0.6 (Applied Biosystems).

The efficiency of the reaction was examined using the standard curves created by serially diluting samples which contain the target sequence. The slope of the line was used to examine the efficiency of the reaction:

$$y = mx + c$$

The reaction efficiency E was calculated as follows:

$$E = (10^{(-1/\text{slope})}) - 1$$

If the slope of the standard curve was -3.2 then the PCR was 100% efficient. With 100% efficiency, a 10X dilution gave a ΔC_t of 3.2 values between each dilution (every 3.2 cycles the amount of amplification was 10-fold higher).

The corrected C_t value was calculated using the equation:

$$\text{Corrected } C_t = C_t + (N_t - C_t') \times E/E'$$

Where C_t = sample (target gene) mean from triplicate, N_t = total reference gene mean from triplicate, C_t' = sample reference gene mean, E = target reaction efficiency and E' = reference reaction efficiency.

Corrected 40- C_t is the total number of PCR cycles (40) minus the corrected C_t value.

Analysis of TaqMan® results was performed using Windows Excel.

2.4.3.8 SYBR® Green assay

2.4.3.8.1 Optimisation of 1-step RT-qPCR (SYBR® Green)

This RT-qPCR assay was performed using a *Power SYBR® Green RNA-to- C_T ™ 1-Step* kit (Applied Biosystems) according to the manufacturer's protocol. A 20 μ l reaction contained 10 μ l Power SYBR® Green PCR Master Mix (2X), 0.4 μ l primer dilution (100-1000 nM final concentration) (Table 2.3), 0.16 μ l RT Enzyme Mix (125X), 5 μ l RNA template and 4.4 μ l nuclease-treated H₂O. Thermal cycling on the ABI 7500 FAST sequence detection system (Applied Biosystems) using Standard Mode was performed as outlined in Table 2.12

Table 2.12. Cycling conditions for 1-step RT-qPCR (SYBR® Green).

Stage	Step	Temperature (°C)	Time
Holding	Reverse transcription	48	30 min
Holding	Activation of <i>Taq</i> polymerase	95	10 min
Cycling (x40)	Denature	95	15 s
	Anneal/Extend	60	1 min
Melt Curve	Denature	95	15 s
	Anneal	60	15 s
	Denature	95	15 s

2.4.3.8.2 Optimisation of 2-step RT-qPCR (SYBR® green)

A cDNA template was generated as described in section 2.4.2 using RNA from DCs stimulated with LPS (200 ng/ml) for 3 h and splenocytes stimulated with Concanavalin A (ConA) (Sigma) (1 µg/ml) for 4 h from 6-week-old J line birds.

This RT-qPCR was performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems). A 20 µl reaction contained 10 µl Power SYBR® Green PCR Master Mix (2X), 2 µl primer dilution (50-900 nM final concentration) (Table 2.3), 2 µl cDNA template and 6 µl nuclease-treated H₂O. Thermal cycling on the ABI 7500 FAST sequence detection system (Applied Biosystems) using Standard Mode was performed as outline in Table 2.13.

Table 2.13. Cycling conditions for 2-step RT-qPCR (SYBR®Green).

Stage	Step	Temperature (°C)	Time
Holding	Activation of <i>Taq</i> polymerase	95	10 min
Cycling (x40)	Denature	95	15 s
	Anneal/Extend	60	1 min
Melt Curve	Denature	95	15 s
	Anneal	60	15 s
	Denature	95	15 s

2.5 TA-cloning

2.5.1 A-tailing PCR products

Purified PCR products were A-tailed using *Taq* polymerase (Invitrogen). The 10 μ l reaction contained 1 μ l 10X PCR buffer, 0.3 μ l 50 mM $MgCl_2$, 10 mM dATP, 7.2 μ l PCR product and 0.5 U *Taq* polymerase. The reaction was incubated at 72°C for 5 min and inactivated by incubation on ice.

A-tailed PCR products were ligated into the TA cloning vectors pGEM®-T Easy and pTarget™ (Promega) (Appendix 2). The 10 μ l reaction mixture contained approximately 50 ng (7.2 μ l) of vector, 3 μ l DNA, 1 U of T4 DNA ligase and 1X T4 DNA ligase buffer and was incubated at RT for 2 h.

2.5.2 TA cloning RT-PCR products

Purified and A-tailed RT-PCR products were ligated into pGEM®-T Easy vector for sequencing or pTarget™ (Appendix 2) for transient recombinant protein expression in COS-7 cells. The ligation reaction contained 1 μ l vector, 3 μ l of the DNA to be cloned, 3 U (1 μ l) of T4 DNA ligase and 5 μ l 2X Rapid Ligation Buffer and was incubated at RT for 2 h.

2.5.3 Transformation and screening

E. coli JM109 competent cells (Appendix 3) were placed on ice to thaw for approximately 5 min. Aliquots of cells (100 μ l) were added to pre-chilled tubes containing 5 μ l of ligation reaction and gently flicked to mix. The ligation/cell mixture was incubated on ice for 20 min and then heat-shocked for 45 s exactly at 42°C before a further 2 min incubation on ice. Room temperature super-optimal broth with catabolite repression (SOC) was then added to give a total of 1 ml per

transformation reaction. The tubes were incubated with shaking at 200 rpm at 37°C for 1 h. Cells were gently agitated to mix and 200 µl of each reaction in duplicate (2 x 200 µl) were plated onto LB plates containing 100 µg/ml ampicillin/0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG)/80 µg/ml X-gal and incubated inverted overnight at 37°C. White coloured colonies are indicative of vector (amp^r) containing an insert, while blue colonies contain empty vector.

2.5.4 Restriction digestion

Restriction digests were carried out using restriction enzymes and buffers (Table 2.14) to release DNA inserts from cloning vectors for positive confirmation of successful TA cloning. DNA digests were performed under conditions recommended by the manufacturer. Standard 10 µl reactions contained 1X final volume buffer (1 µl), 1 µl of DNA, 1 µl of enzyme and 7 µl of nuclease-free H₂O and were incubated at 37°C for 1.5 h.

Table 2.14. Restriction enzymes used in this study.

Restriction Enzyme	Cut Site	Reaction Buffer	Manufacturer
<i>NotI</i>	GC/GGCCGC	React 3	Invitrogen
<i>EcoRI</i>	G/AATTC	Buffer H	Promega

2.6 DNA sequencing

Sequencing was performed by either DNA Sequencing and Services at the University of Dundee on an Applied Biosystems 3730 DNA analyser or GATC Biotech AG, Cologne, Germany on an Applied Biosystems 3730xl analyser.

2.7 Isolation and stimulation of primary cells

Cells were isolated from the spleen, thymus, bursa of Fabricius and bone marrow from the femurs and tibias of chickens for stimulation or differentiation and subsequent RNA extraction. Tissues were removed and stored in PBS on ice.

2.7.1 Splenocytes

A single cell suspension was prepared in a 50 ml Falcon tube containing 15 ml cold PBS. The relevant tissue was cut in half with scissors, and mashed through a 70 μ m cell strainer using the end of a syringe plunger. Eight millilitres Histopaque 1.077 (Sigma) were underlaid beneath the single cell suspension using a 10 ml pipette and centrifuged at 400 x *g* for 20 min at RT with the centrifuge brakes off. Lymphocytes were drawn off the density gradient interface with a wide mouth pipette and washed with 20 ml PBS before centrifugation at 300 x *g* for 10 min at 4°C. The wash step was repeated and the cells re-suspended in complete Dulbecco's modified eagle medium (DMEM) (Appendix 1) at 5 x 10⁶ cells/ml following a viable cell count with Trypan blue (Life Technologies) and a haemocytometer. Cells (5 x 10⁵) were added to each well of a U-bottomed 96-well plate. The splenocytes were stimulated with ConA at 1 μ g/ml, for 2, 4, 6, 12, 18 and 24 h. Cells were harvested post-stimulation by centrifugation at 250 x *g* for 10 min, media were removed with a pipette and triplicate wells of cells re-suspended in 350 μ l of buffer RLT. RNA was isolated from cell lysates using the method described in section 2.3.2.1.

2.7.2 Thymocytes

A single cell suspension was prepared by mashing the tissue through a cell strainer as in section 1.3.10.1. Cells were pelleted by centrifugation at 250 x *g* for 10 min at RT.

The supernatant was poured off and the cell pellet re-suspended in 20 ml of complete DMEM. A viable cell count using Trypan blue and a haemocytometer was performed and cells re-suspended at 5×10^6 cells/ml. Cells (5×10^5) were added to each well of a U-bottomed 96-well plate. Thymocytes were stimulated with phytohaemagglutinin (PHA-P) (Sigma) at 25 μ g/ml for 2, 4, 6, 12, 18 and 24 h. The cells were harvested as described in section 1.3.10.1. RNA was isolated as in section 2.3.2.1.

2.7.3 Bursal Cells

Bursal cells were prepared as described in section 1.3.10.2 and stimulated with phorbol 12-myristate 13-acetate (PMA) (Sigma) and ionomycin (Sigma) at 800 ng/ml and 100 ng/ml respectively for 2, 4, 6, 12, 18 and 24 h. Bursal cells were also harvested as described in section 1.3.10.1 and RNA isolated as in section 2.3.2.

2.7.4 Isolation and differentiation of bone marrow-derived dendritic cells

Isolation and differentiation of bone marrow-derived dendritic cells (BMDCs) was based on the method described by Wu *et al.* (2010).

Both ends of the femur and tibia bones were cut off and the bone marrow flushed out into a Petri dish using a 21G needle, 10 ml syringe and sterile PBS. A cell suspension was created by gently mixing up and down using a pipette. A single cell suspension was created by passing the cells through a 70 μ m cell strainer into a 50 ml Falcon tube. Cells were pelleted by centrifugation at 500 x g for 10 min at RT. The supernatant was discarded and the cell pellet re-suspended in 15 ml sterile PBS. Fifteen millilitres Histopaque 1.077 were underlaid beneath the single cell suspension using a 20 ml pipette and centrifuged at 1200 x g for 20 min at RT with the centrifuge brakes off. Cells were drawn off the density gradient interface with a

wide-mouthed pipette, transferred to a Universal and washed with 20 ml PBS before centrifugation at 300 x *g* for 10 min at 4°C. The supernatant was discarded and cells re-suspended in 10 ml pre-warmed complete Roswell Park Memorial Institute (RPMI) medium (Appendix 1). A viable cell count was performed using Trypan blue and a haemocytometer and the concentration of cells adjusted to 2×10^6 cells/ml.

Recombinant rhIL-4 and rhGM-CSF were transiently expressed from COS-7 cells as described in section 2.8.4. Three millilitres of cell suspension supplemented with 7.5 µl rhGM-CSF (1/400) and 15 µl rhIL-4 (1/200) were added to each well of 6-well plates and incubated at 41°C, 5% CO₂ for 6 d. Fresh complete RPMI medium supplemented with rhGM-CSF and rhIL-4 replaced 75% of the old media every 2 days.

2.7.5 *Stimulation of bone marrow-derived dendritic cells*

Chicken BMDCs were isolated and differentiated as described in sections 2.7.4. On day 6 of differentiation, all media were carefully removed from each well with a pipette without disrupting the adherent cells, and was replaced with 3 ml of complete RPMI media supplemented with ex-COS rhIL-23 (1:100 or 1:250), LPS (25-200 ng/ml) or recombinant chicken pcDNA3 protein (1:100 or 1:250) for 3, 6 and 24 h. Cells were harvested by adding 5 drops of 0.5 M EDTA from a pastette to each well. The plate was gently agitated and incubated at RT for 3-5 min. The cells were pipetted up and down to ensure all adherent cells were removed and transferred to a Universal. The cells were pelleted by centrifugation at 300 x *g* for 5 min and the supernatant discarded. The cell pellet was re-suspended in 700 µl buffer RLT and

transferred to a 1.5 ml Eppendorf tube for storage at -20°C. RNA was isolated from cell lysates as described in section 2.3.2.1.

2.8 Cell culture

2.8.1 *Resurrection of cryopreserved COS-7 cell line*

Cells (Appendix 3) were retrieved from liquid nitrogen and thawed in a 37°C water bath. Cells were washed with 20 ml of warmed growth DMEM (Appendix 1), pelleted by centrifugation at 216 x *g* for 5 min and re-suspended in growth DMEM. A T75 culture flask was seeded with 7.5×10^5 COS-7 cells. The cells were incubated at 37°C, 5% CO₂ and checked regularly.

2.8.2 *General maintenance of COS-7 cell line*

COS-7 cells were grown adherently until 90% confluent and then passaged, typically, every 2-3 days. Trypsin/versene solution (Gibco) at a 1/10 dilution was prepared. Old media were removed and cells washed twice with 15 ml pre-warmed PBS. Five ml of trypsin/versene solution was added and discarded. Another 5 ml of trypsin/versene solution was added and the flask incubated at 37°C, 5% CO₂ for 5 min. The flask was tapped on the bench and 15 ml of growth DMEM added to inactivate the enzyme. The cells were pelleted by centrifugation at 216 x *g* for 5 min and the supernatant discarded. The cells were counted using Trypan blue and a haemocytometer. T75 flasks were re-seeded with 7.5×10^5 COS-7 cells. The cells were checked regularly and passaged every 3-4 days.

2.8.3 *Transfection of COS-7 cells*

Twenty-four hours prior to transfection, COS-7 cells were seeded at 6×10^6 cells per T75 flask and incubated overnight at 37°C, 5% CO₂. Fifteen ml of serum-free growth DMEM containing 112.5 µg of endotoxin-free plasmid DNA, 150 µl 10 M chloroquine (Sigma) and 90 µl of 100 mg/ml DEAE/Dextran (Sigma) were used per transfection. The cell layer was washed twice with PBS and the DNA/DEAE complex added. The cells were incubated at 37°C, 5% CO₂ for 3 h. The DNA/DEAE complex was removed and the cell layer washed with PBS. Ten percent dimethyl sulfoxide (DMSO) (Sigma) in PBS was added for 2 min, and then replaced with 5 ml of growth DMEM. The cells were incubated at 37°C, 5% CO₂ overnight. The medium was changed for 15 ml serum-free growth DMEM. The supernatant was harvested after 3 d, centrifuged at 216 x g for 5 min and stored at 4°C. The cells were harvested after 3 d using trypsin/versene and re-suspended in appropriate amount of buffer RLT (Qiagen) for RNA isolation (section 2.3.2.1).

2.8.4 *Transient recombinant protein expression in COS-7 cells*

COS-7 cells were transiently transfected with chicken cDNA clones (Appendix 3) by the DEAE/dextran method. COS-7 cells were trypsinised, counted and re-seeded at 6×10^6 cells/75 cm² flask. Cells were then cultured for 18-24 h at 37°C, 5% CO₂. For each separate cDNA, a transfection mix for a 75 cm² flask was made containing 15 ml serum-free growth DMEM (Appendix 1), 112.5 µg DNA, 258 µg/ml chloroquine and 600 µg/ml DEAE/dextran. Cells were washed with 2 x 10 ml warm PBS, followed by the addition of the transfection mix. Cells were incubated for 3 h at 37°C, 5% CO₂ to facilitate DNA uptake. The transfection mix was discarded and cells were washed with another 10 ml warm PBS. Next, a solution of PBS containing

10% DMSO was added for 2 min to shock the cells, increasing the efficiency of the transfection. The PBS/DMSO was then discarded and replaced with 15 ml growth DMEM. After incubating the cells for 16-24 h at 37°C, 5% CO₂, growth DMEM was changed for serum-free growth DMEM. Cell supernatants were harvested 72 h later.

2.9 IL-23 bioassay

2.9.1 Optimising the IL-23 bioassay

Preliminary assays were carried out to determine the optimal conditions and methods to quantify the bioactivity of rchIL-23, as described by Aggarwal *et al.* (2003). Lymphocytes were isolated over Histopaque 1.077 from the spleen of 6-week-old J line birds, washed and re-suspended at $5-10 \times 10^6$ cells/ml in complete DMEM, as described in section 2.7.1. The cell suspension was added to 96-well flat-bottomed plates (100 µl/well) containing different dilutions of ex-COS rchIL-23 (1/10-1/500) and ex-COS rchIL-2 (1/100-1/500) or purified rchIL-23 (Dundee Cell Products) (0.1-1000 ng/ml) in a final volume of 200 µl/well. ConA (1 µg/ml) was used as a positive control and medium alone was used as a negative control. Assays were conducted in triplicate. Cells were incubated at 41°C in 5% CO₂ for 6 h, 1, 2, 3 and 4 d with ex-COS rchIL-23 and rchIL-2 and 1, 2 and 3 d with purified rchIL-23 alone (Dundee Cell Products). RNA was isolated as described in section 2.3.2.1 to measure expression of chIL-17A, chIFN γ and chIL-2 transcripts by TaqMan®.

2.9.2 Optimised IL-23 bioassay conditions

Lymphocytes were isolated over Histopaque 1.077 from the spleen of 6-week-old J line birds, washed and re-suspended at 5×10^6 cells/ml in complete DMEM, as described in section 2.7.1. The cell suspension was added to 96-well U-bottomed

plates (100 μ l/well) containing purified rchIL-23 (Dundee Cell Products) (0.1-1000 ng/ml) and ex-COS rchIL-2 (1/250) in a final volume of 200 μ l/well. ConA (1 μ g/ml) was used as a positive control and medium alone was used as a negative control. Assays were conducted in triplicate. Cells were incubated at 41°C in 5% CO₂ for 6 h, 1, 2, 3, 4 and 6 d. RNA was isolated as described in section 2.3.2.1 to measure expression of cytokine transcripts by TaqMan® assay.

2.10 Dot-blot assay

Dot-blot assays were used to investigate if mouse anti-chicken IL-12 p40 Abs (Appendix 3) recognise rchIL-23. A Hibri-Dot 96 device (Whatman) was used to isolate protein on nitrocellulose membrane. The device was assembled by laying a membrane-support plate on a waste-reservoir plate, followed by a sheet of nitrocellulose membrane to cover every hole on the support plate. Finally, a cover plate with 96 wells and a sample reservoir were carefully placed on the membrane. With all three plates in the correct position, they were tightened by 8 screws.

Protein samples (50 μ l) were added to the wells on the cover plate of the Hibri-Dot 96 and drawn onto the membrane by vacuum. The membrane was removed, washed in buffer PBS and Tween20 (PBST) for 5 min and blocked in 0.2% casein/PBS solution for 1 h. The membrane was washed twice more in PBST for 2 min. The membrane was then transferred into a mouse anti-chicken IL-12 p40 Ab solution (AV134, AV135 or AV136), 1/500 dilution in 0.2% casein/PBS, and incubated for 1 h at room temperature. After three washes with PBST, 5 min per wash, the secondary antibody, goat anti-mouse IgG1-HRP, diluted 1/1000 in 0.2% casein/PBS, was added to the membrane and incubated for 1 h. The membrane was washed 3 times as before

and then additionally for 2 further washes in PBST for 10 min each. The nitrocellulose membrane was then washed for 1 min in Milli-Q water and dried with tissue paper. All wash and incubation steps were carried out on a rocking shaker.

The binding secondary Ab was detected using enhanced chemiluminescence (ECL) Western Blotting Detection Reagents (GE Healthcare), as per the manufacturer's protocol. Equal volumes of luminol substrate and peroxide solution from the ECL kit were directly mixed before use, homogeneously distributed on the membrane, incubated for 5 min and dried with tissue paper. The membrane was visualised and a digital image captured on a G:Box (Syngene).

2.11 Differentiation of CD4⁺ T cells

2.11.1 *Fluorescence-activated cell sorting (FACS)*

Cells were analysed and sorted by flow cytometry using a FACS Aria III instrument (BD Becton Dickinson) by Bob Fleming (The Roslin Institute).

Lymphocytes from the spleens of 7-9-week-old J line birds, processed as described in section 2.7.1, were adjusted to 1×10^8 cells/ml. For immunostaining, cells were aliquoted into 5 x 1 ml aliquots in 15 ml Falcon tubes. All Abs were used at a 1/200 dilution, therefore 5 µl of each Ab were added to each 1 ml aliquot of cells. For negative cell sorting, cells were stained with the following combinations of cell surface marker Abs and fluorochromes: TCR-1-FITC (fluorescein isothiocyanate), KUL01-FITC, CD8α-PE (phycoerythrin) and Bu-1-A647 (Alexa Fluor 647) (all Southern Biotech). For positive cell sorting, cells were stained with CD4-PE (Southern Biotech). Sytox[®] blue dead cell stain (LifeTech) was used to measure

viability of the cells. The cells were incubated at RT in the dark for 20 min. The cells were washed 3 times with 2 ml complete DMEM and pelleted by centrifugation at $300 \times g$ for 5 min. After the final wash step, each aliquot of cells was re-suspended in 2 ml complete DMEM and filtered through a $35 \mu\text{m}$ filter using a 12 x 75 sterile polypropylene tube with built-in sieve top (Becton Dickinson) to create a single cell suspension for FACS. The sorted cells were eluted into complete DMEM and pelleted by centrifugation at $300 \times g$ for 15 min. The cell pellets were re-suspended in $500 \mu\text{l}$ complete DMEM and pooled together for a viable cell count using Trypan blue and a haemocytometer. Data analysis was performed using FACSDiva software.

2.11.2 *Magnetic-activated cell sorting (MACS)*

Lymphocytes were isolated over Histopaque 1.077 from the spleens of 7-9-week-old J line birds, washed and re-suspended in 5 ml complete DMEM, as described in section 2.7.1, before performing a viable cell count using Trypan blue and a haemocytometer. The cells were pelleted by centrifugation at $300 \times g$ at 4°C for 10 min and re-suspended in 12.5 ml MACS buffer ($2.5 \text{ ml per } 10^8$ cells). Mouse anti-chicken CD4 Ab (unconjugated, Southern Biotech) was used for immunostaining at a 1/500 dilution. The cells were incubated at 4°C for 30 min in the dark, washed with 50 ml MACS buffer and pelleted by centrifugation as before. The cells were then re-suspended in 4 ml MACS buffer ($800 \mu\text{l per } 10^8$ cells) and $500 \mu\text{l}$ of goat anti-mouse IgG MACS® MicroBeads (Miltenyi Biotec) were added ($200 \mu\text{l per } 10^8$ cells), which bind to the Fc domains of the mouse anti-chicken monoclonal CD4 Ab. The cell solution was incubated at 4°C for 15 min in the dark with continuous mixing. The cells were washed again as before and the cell pellet re-suspended in 2.5 ml MACS buffer.

Magnetic separation of cells was performed using an LS Column and a MidiMACS™ Separator (Miltenyi Biotec). After attaching an LS Column to the MidiMACS™ Separator, 3 ml of MACS buffer were allowed to run through the LS Column to equilibrate it. The cell solution was applied to the LS Column, 3 ml at a time, and allowed to run through. The flow-through fraction contained the negatively depleted labelled cells. To collect the CD4⁺ cells, the LS Column was removed from the MidiMACS™ Separator and the retained cells were eluted with MACS buffer as the positively selected cell fraction. Five millilitres of MACS buffer were applied to the LS Column and the plunger used to apply force through the syringe. This was repeated a second time. A viable cell count was performed using Trypan blue and a haemocytometer. The sorted cells were washed in 10 ml complete DMEM and pelleted by centrifugation at 300 x g for 10 min. The cell pellet was re-suspended in complete DMEM at 5 x 10⁶ cells/ml.

Post-MACS, cells were analysed by flow cytometry using a FACSCaliber (Becton Dickinson) by Lonneke Vervelde (The Roslin Institute). Data analysis was performed using FlowJo software (TreeStar Inc., Ashland, USA).

2.11.3 Stimulation of CD4⁺ cells towards a Th17 phenotype

CD4⁺ T cells from splenocytes isolated from 7-9-week-old J line birds were positively or negatively sorted by FACS or MACS as described in sections 2.11.1 and 2.11.2.

U-bottomed 96-well plates were coated with mouse anti-chicken CD3 and CD28 monoclonal Abs (mAbs) to activate CD4⁺ T cells (50 µl/well). Antibodies were used at 1 µg/ml and a 1/250 dilution respectively and diluted in PBS. The Ab-coated

plates were incubated at 41°C, 5% CO₂ for 3 h. Plates were washed 3 times in sterile PBS to remove unbound Ab.

The cell suspension of CD4⁺ cells (1-5 x 10⁶ cells/ml dependent on total cell number after sorting) was added to 96-well U-bottomed plates (50 µl/well) coated with mouse anti-chicken CD3 and CD28 mAbs in a final volume of 100 µl/well with rhTGF-β4, rhIL-6 and rhIL-12 as a positive control, diluted 1/125, 1/250 and 1/500 in complete DMEM. ConA (1 µg/ml) was also used as a positive control and medium alone was used as the negative control. Assays were conducted in duplicate. Cells were incubated at 41°C, 5% CO₂ for 3-5 d. RNA was isolated as described in section 2.3.2.1 to measure expression of chIL-17F transcripts by TaqMan® assay.

2.12 Statistical Analyses

All statistical analyses were carried out using Minitab 17. Statistical significance was determined with a P value of < 0.05.

2.12.1 *t* test

A standard one-sample *t* test was performed on normally distributed data to determine if there was a significant up- or down-regulation in fold change of cytokine mRNA expression compared to a fold change of one at each time point. The residuals were checked for normality and all cytokine responses required log transformation to normalise the residuals.

2.12.2 *Mann-Whitney U* test

A Mann-Whitney U test was performed on abnormally distributed data from the *S. aureus* experiment to determine if there was a significant difference in chIL-17A

mRNA expression in the bone marrow between wild-type, mutant and control groups at 11 dpi.

2.12.3 ANOVA (1-way)

A one-way analysis of variance (ANOVA) was performed on data from the IBDV experiment to determine if there was a significant difference between cytokine mRNA levels at different time points. The residuals were checked for normality and all cytokine responses required log transformation to normalise the residuals. If overall differences were found, a standard post-hoc Tukey test was used as a multiple comparison method to determine the time points at which cytokine mRNA expression was significantly different to each other.

2.12.4 ANOVA (2-way)

A two-way ANOVA with interactions was performed on data from the *S. aureus* experiment to determine if there was a significant difference in fold change of cytokine mRNA expression of the mutant and wild-type groups compared to uninfected controls and also a significant difference in mRNA expression between the time points. The interaction between infected group and time was also statistically analysed for significance. The residuals were checked for normality and all cytokine responses required log transformation to normalise the residuals. If the most complicated interaction of time and infected group was not significant, it was removed from the model and the model re-run to analyse the lesser complicated interactions of time and infected group alone. This step-wise approach elucidated the final minimal model and any significant interactions between groups of data.

2.12.5 Regression analysis

Standard linear regression was used to show the relationship between cytokine mRNA expression and time during *H. meleagridis* infection. The residuals were checked for normality and required no further normalisation. Examination of the raw data revealed the possibility of a non-linear relationship between cytokine mRNA expression and time. The regression model (linear, quadratic or cubic) which best described the relationship was chosen. Initially, a cubic relationship was applied, if the cubic term was not significant, a more simple quadratic relationship was applied. If the quadratic term was not significant, the simplest linear relationship was applied and tested for significance. Significance values, R^2 values and 95% confidence intervals (CI) were calculated for each model.

2.12.6 Linear mixed effect model

A linear mixed effect model was used to show the relationship between cytokine mRNA expression and time in stimulated spleen, thymus and bursal cells. This analysis accounted for the random effect of the repeated use of individual birds. The residuals were checked for normality and all cytokine responses required log transformation to normalise the residuals. Examination of the raw data revealed the possibility of a non-linear relationship between cytokine mRNA expression and time. Initially, a quadratic relationship was applied, if the quadratic term was not significant, the simpler linear relationship was applied and tested for significance. The regression model (linear or quadratic) which best described the relationship was chosen.

2.12.7 *Mixed effect model*

A mixed effect model was used to show the relationships between concentration of IL-23-stimulation and time of stimulation on cytokine mRNA expression in rchIL-23-stimulated splenocytes. This analysis accounted for the random effect of the repeated use of individual birds as well as considering an interaction effect between IL-23-stimulation and the addition of IL-2. The residuals were checked for normality and all cytokine responses required log transformation to normalise the residuals. Examination of the raw data revealed the possibility of a non-linear relationship between cytokine mRNA expression and both concentration of IL-23-stimulation and time of IL-23-stimulation. Initially, a quadratic relationship was applied and an interaction effect of IL-23 and IL-2 co-stimulation was tested for. If there was no significant interaction effect in the quadratic relationship, the simpler linear relationship and an interaction effect of IL-23 and IL-2 co-stimulation was analysed. If there was no interaction effect of the addition of IL-2, the quadratic term was tested for significance and if not significant, the simpler linear relationship applied and tested for significance. The regression model (linear or quadratic) which best described the relationship was chosen.

2.12.8 *Analysis of variance*

Analysis of variance was used to analyse data from the MD experiment. A multivariate approach was used whereby the maximum complicated model was applied to the data and the most complicated interaction (days post infection, line of bird and infected/uninfected samples) was tested for significance. The residuals were checked for normality and the data required no further normalisation. If the most complicated interaction was not significant, it was removed from the model and the

model re-run to analyse the next least complicated interactions. The same rules were subsequently applied to the data, using a step-wise approach, to elucidate the final minimal model and any significant interactions between groups of data.

To graphically display the MD data, boxplots were used. The interquartile range box represents the middle 50% of the data (first and third quartiles) and the band inside the box is the median (second quartile). If the sample has more than 3 data points, then the upper whisker represents the upper 25% of the distribution and the lower whisker represents the lower 25% of the distribution.

Chapter 3 Cloning and biological characterisation of chIL-23 p19

3.1 Introduction

IL-23 is a member of the IL-12 family of cytokines and is a heterodimer of the p19 and p40 subunits (Oppmann *et al.*, 2000). In humans, the IL-23 p19 gene is located on chromosome 12 (Flicek *et al.*, 2014) and with p40 forms a pro-inflammatory cytokine with a key role in the development of Th17 cells. IL-23 is expressed as a disulphide-linked heterodimer, primarily by DCs and tissue-resident macrophages (Basso *et al.*, 2009). The main focus of research on the role of IL-23 in mice and humans has been its involvement with Th17 cell differentiation and consequently its role in autoimmunity.

The formation of biologically active IL-23 requires the synthesis of both subunits within the same cell. As a result, purified IL-23 p19 is biologically inert *in vitro* and is poorly secreted in the absence of IL-23/IL-12 p40 (Wiekowski *et al.*, 2001). Several factors are known to regulate the expression of IL-23 in mammals but the exact mechanisms that control IL-23 production remain incompletely understood. However, the stimuli to induce mammalian IL-23 expression are somewhat characterised. PRR activation leads to preferential expression of IL-23 at mucosal surfaces (Yannam *et al.*, 2012) while TLR2 agonists, such as peptidoglycan, are powerful inducers of IL-23 expression (Re and Strominger, 2001). In addition, stimulation of TLR3 and TLR7 augment the expression of IL-23 p19 in macrophages (Al-Salleeh and Petro, 2007). LPS is also an inducer of IL-23 in human monocytes, macrophages and DCs (Roses *et al.*, 2008). The interaction of CD40 and CD40L

increases production of IL-23 and generates a positive feedback loop, further augmenting IL-23 production (Sender *et al.*, 2010).

IL-23 exerts its biological effects through its interaction with the IL-23R, (which is discussed in more detail in Chapter 5), which is expressed on innate and adaptive immune cells. In addition to Th17 cells, IL-23R expression is also found on $\gamma\delta$ T cells and ILCs (Buonocore *et al.*, 2010; Sutton *et al.*, 2009). The differentiation of Th17 cells is not dependent on IL-23 (Korn *et al.*, 2009; McGeachy *et al.*, 2009) but it is fundamental for maintenance of Th17 cells *in vitro* and *in vivo* (Chen *et al.*, 2011). In mammals, IL-23 drives expression of the effector cytokines IL-17A, IL-17F, IL-22 and GM-CSF in Th17 cells (Aggarwal *et al.*, 2003; Codarri *et al.*, 2011; Ivanov *et al.*, 2006; Liang *et al.*, 2006).

IL-23 p19 has been identified in the sheep (Gossner *et al.*, 2012), horse (Tompkins *et al.*, 2010), cattle (Chen *et al.*, 2009) and pig (Kokuho *et al.*, 2012) as well as zebrafish (Holt *et al.*, 2011). The biological role of IL-23 is predicted to be the same in these species but this is yet to be demonstrated.

For biological analysis of IL-23 in birds, nt and aa sequences of the ligands and receptors in the IL-23 system are required, however, until this study, no data were available on the primary structures of chIL-23 p19 and the chIL-23R. This is because the IL-23 p19 subunit is “missing” from, and the IL-23R gene is misannotated, in the chicken genome. As a result, the initial aim of this Chapter was to identify the gene for IL-23 p19 in the chicken and consequently investigate its expression and bioactivity compared to mammals. Using bioinformatic analysis, the chIL-23 p19 protein-coding cDNA sequence was identified and its mRNA expression measured in

chicken tissues and stimulated lymphoid cells using RT-qPCR. To study the biological role of chIL-23 compared to mammals, a chIL-23 flexi-construct was designed to express full-length rchIL-23 and its bioactivity demonstrated. Because of the shared p40 subunit of IL-23 and IL-12, chIL-12 p40 mAbs, which recognise chIL-12 p70 (Balu *et al.*, 2011), were used to investigate if they also recognise rchIL-23.

To summarise, the aims of this Chapter were to identify and clone chIL-23 p19, explore the structural and functional similarities and differences between avian and mammalian IL-23 p19 as well as investigate the mRNA expression patterns of chIL-23 p19 in chicken tissues and stimulated cells.

3.2 Methods

General methods are outlined in Chapter 2. Additional methods and alterations are detailed here.

3.2.1 Cloning of chIL-23 p19 cDNA

Chicken IL-23 p19 is not currently annotated in the chicken genome (Galgal 4.0, Ensembl). Forward and reverse primers were designed to a partial nt sequence of chIL-23 p19 (Table 2.1 IL-23_F1 and IL-23_R1). This was an expressed sequence tag (EST) of chIL-23 p19 (Accession Number CK613546.1) previously identified in the data that was not included in the genome build by Mark Gibson (Institute for Animal Health). A platypus IL-23 aa sequence was used in a Protein Basic Local Alignment Search Tool (BLASTP) (www.ncbi.nlm.nih.gov/blast) search of the National Centre for Biotechnology Information (NCBI) EST database, identifying a

chicken EST (Accession Number CK613546.1), which represented a putative novel chicken IL-23 p19 gene. Following successful PCR amplification (section 2.4.3.2) of cDNA, corresponding to the EST sequence, from stimulated chicken splenocyte RNA (section 2.7.1), a partial length chIL-23 p19 cDNA clone was TA-cloned into pGEM®-T Easy and sequenced as described in section 2.5.

The chIL-23 p19 EST sequence was used as a probe to screen the NCBI nt database and located a predicted nt sequence (LOC100858964) recently added to the database. Primer design for amplification of chIL-23 p19 (Table 2.1 IL-23F_F2 and IL-23_R3) was based on the predicted sequence (LOC100858964) and RNA isolated from chicken DCs stimulated with LPS for 4 h was used to generate cDNA for PCR (section 2.7.5). The cDNA clone was TA-cloned into pGEM®-T Easy and sequenced. Further bioinformatic characterisation of chIL-23 p19 nt and aa sequences was carried out to confirm the gene's similarity to mammalian IL-23 p19 sequences.

3.2.2 chIL-23 flexi-construct design

A single chain chIL-23 molecule (chIL-23 flexi-construct) was designed (Figure 3.1), based on the mammalian IL-12 and IL-23 flexi-constructs (Belladonna *et al.*, 2002; Lieschke *et al.*, 1997) for recombinant protein expression. Dundee Cell Products generated the chIL-23 flexi-construct, which was cloned into the vector pcDNA3.1 (Appendix 2) and purified recombinant protein expressed in a mammalian cell line (COS-7).

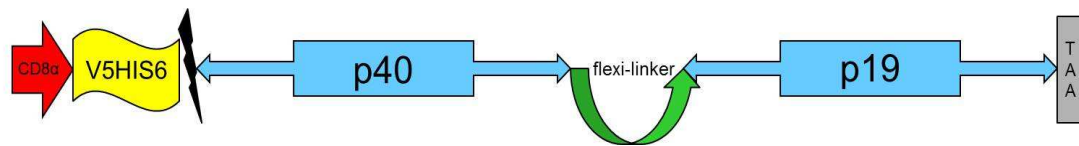


Figure 3.1. Chicken IL-23 flexi-construct design. The mouse CD8 α signal peptide is identified by the red arrow. The V5HIS6 tag is indicated in the yellow wave. A precision protease site is represented by the black lightening symbol. The p40 mature protein follows the precision protease site, flexi-linked to the p19 mature protein sequence, followed by a stop codon.

3.3 Results

3.3.1 Identification and cloning of chIL-23 p19

The EST (Accession Number CK613546.1) was a 229 nt sequence, which was lacking both a start and stop codon and a polyadenylation (polyA) (AATAAA) signal. Translation and alignment, using ClustalX, of this EST to human, mouse and cattle aa sequences showed high identity across a number of residues, including 3 conserved cysteine residues (Figure 3.2).

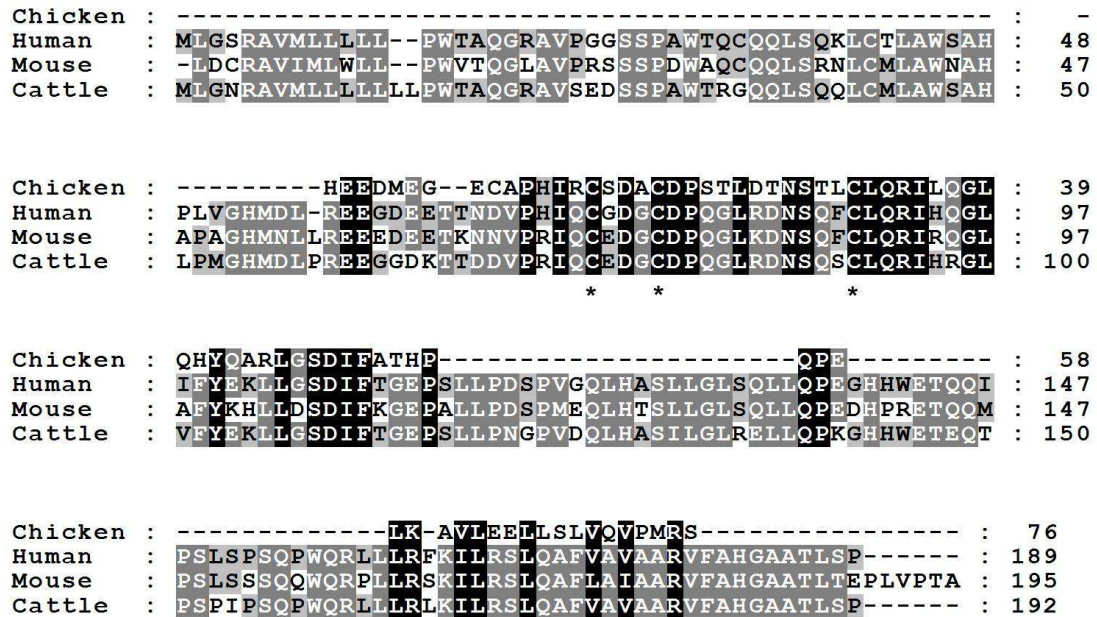


Figure 3.2. Amino acid (aa) alignment of the chIL-23 EST with human, mouse and cattle IL-23 p19 aa sequences. Shaded areas represent conservation of aa similarity, the darker the shading the more conserved the residue is across the species. Conserved cysteine residues are identified by an asterisk.

Using the predicted chIL-23 p19 EST as a probe in the NCBI nt database, a predicted nt sequence for full-length chIL-23 p19 (LOC100858964) was identified. The predicted nt sequence for chIL-23 p19 is located in an unmapped area of the chicken genome. Translation and alignment to human, mouse and cattle IL-23 p19 showed the EST sequence had an identity of 24.1% (Figure 3.3), and was much longer than its mammalian counterparts.

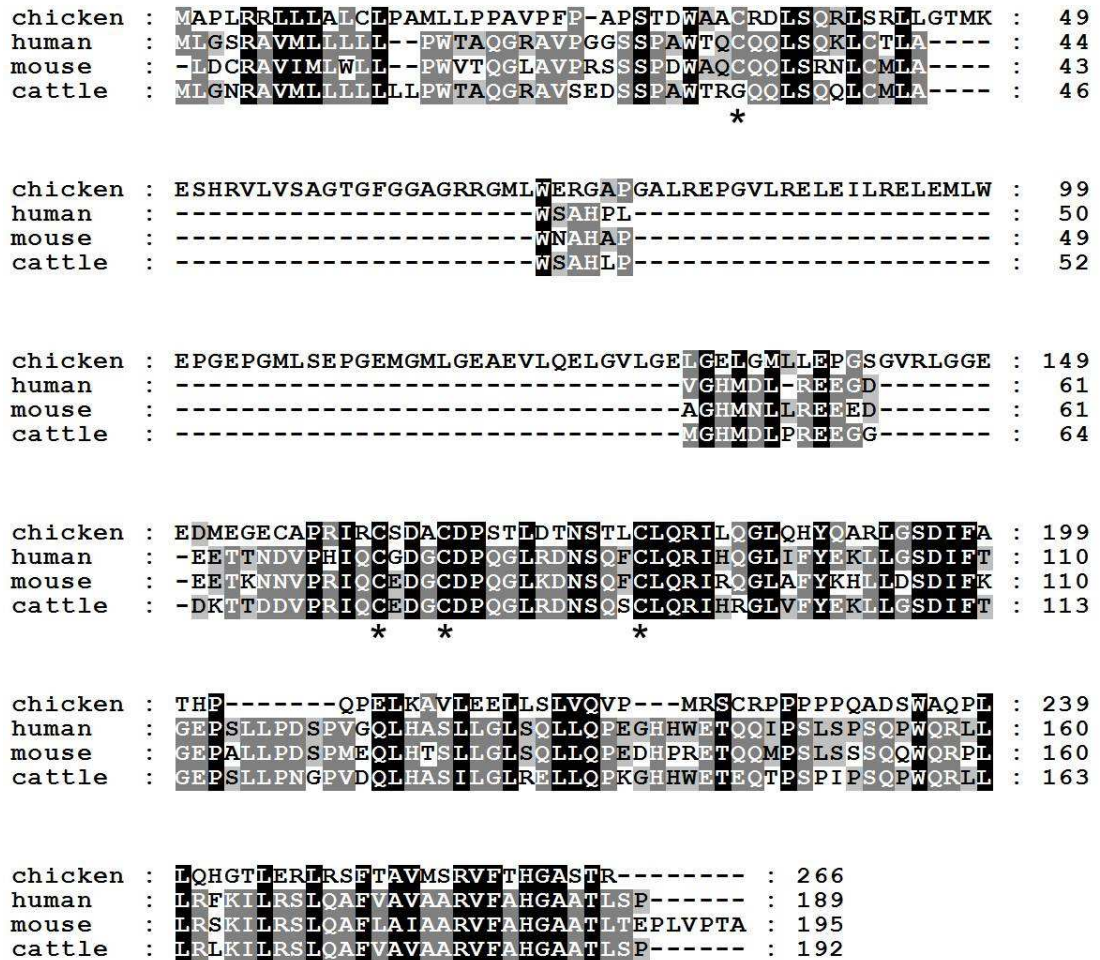


Figure 3.3. Comparison of the predicted chIL-23 p19 (LOC100858964) aa sequence with human, mouse and cattle IL-23 p19 aa sequences. Shaded areas represent conservation of aa similarity, the darker the shading the more conserved the residue is across the species. Conserved cysteine residues are identified by an asterisk.

Based on the predicted nt sequence (LOC100858964), primers IL-23_F2 and IL-23_R3 (Table 2.1) were designed to clone cDNA encoding the predicted full-length chIL-23 p19, using cDNA prepared from LPS-stimulated BMDCs as a template. The predicted length of the amplified chIL-23 p19 cDNA product was 801 nt. However, mammalian IL-23 p19 nt sequences are approximately 500 nt in length. Figure 3.4 shows the product amplified with primers IL-23_F2 and IL-23_R3. It is approximately 500 nt in size and was TA-cloned into pGEM®-T Easy and

transformed into *E. coli* JM109 competent cells for subsequent plasmid isolation and sequencing.

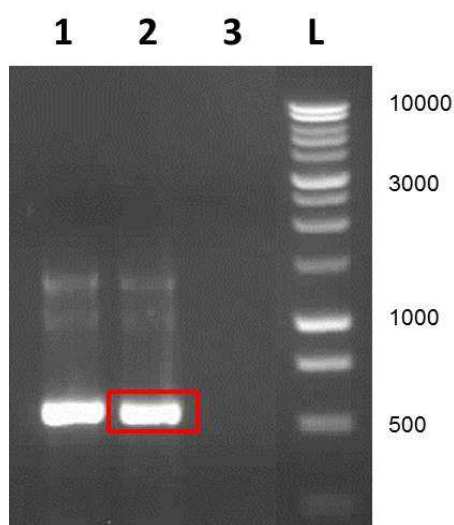


Figure 3.4. RT-qPCR amplification of predicted chIL-23 p19 cDNA. Lane 1: cDNA from LPS-stimulated (2 h) BMDCs. Lane 2: cDNA from LPS-stimulated (4 h) BMDCs. Lane 3: negative control (H₂O). Lane L: GeneRuler™ 10 kb DNA Ladder (Thermo Scientific). Red box highlights PCR product selected for TA cloning and sequencing.

Sequencing of multiple clones confirmed the nt sequence of chIL-23 p19 (Figure 3.6). Comparison of the confirmed and predicted (LOC100858964) chIL-23 p19 nt sequence, using the intron splicing consensus (GT/AG), identified the extra sequence in LOC100858964 as incorrectly predicted, and likely to be intronic sequence (Figure 3.5).

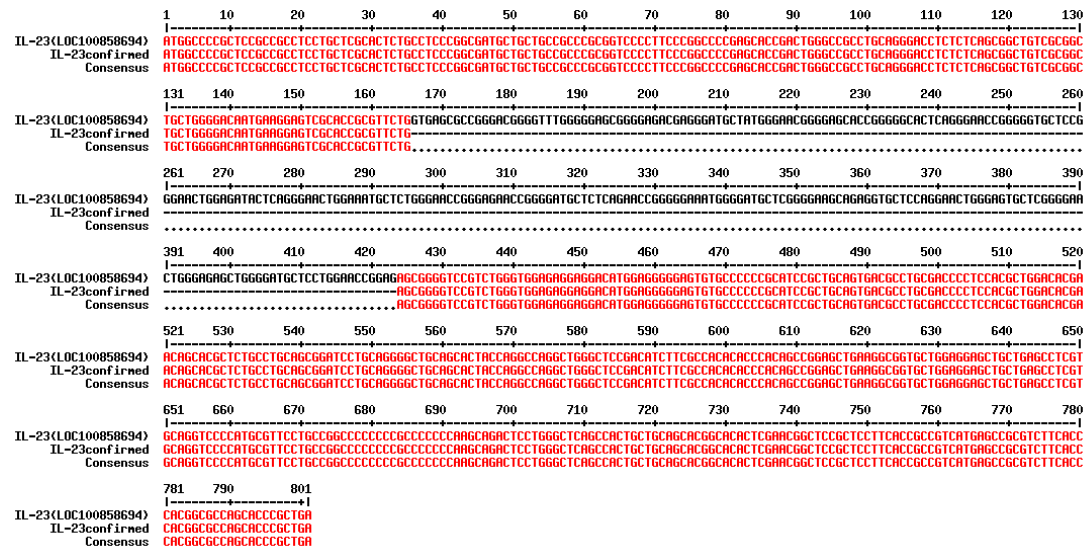


Figure 3.5. Nucleotide sequence alignment of the predicted chIL-23 p19 (LOC100858694) and confirmed chIL-23 p19. Identical nt sequence is red. Incorrectly annotated (presumed) intronic nt sequence is black.

3.3.2 Structural features of chIL-23 p19

The full-length chIL-23 p19 nt sequence was translated and the aa sequence further characterised *in silico*. The chIL-23 p19 cDNA encodes a predicted protein of 180 aa (Figure 3.6), with a putative 23 aa signal peptide identified using SignalP3.0, resulting in a predicted secreted mature protein of 157 aa. The signal peptide of human IL-23 p19 is 19 aa, resulting in a secreted mature protein of 170 aa and identifying the predicted chIL-23 p19 signal sequence cleavage site to be similar to that of mammals. When aligned with mammalian IL-23 p19 sequences, chIL-23 p19 has an aa identity of 42.8%. Chicken IL-23 p19 is very similar in length to the mammalian IL-23 p19 sequences. Further, there is 41.6%, 38.9%, 53.3%, 40.6%, 41.1%, 42.2% and 23.9% aa similarity between chIL-23 p19 and human, mouse, pig, sheep, cattle, horse and zebrafish IL-23 p19 respectively. There are seven cysteine residues in chIL-23 p19, four of which are conserved with mammals. A cysteine residue in human IL-23 p19, which interacts with p40, and a tryptophan residue,

which interacts with the IL-23R, are both conserved between the chicken and mammals. The location of four α -helices (A, B, C and D) determined for human and mouse IL-23 p19 are shown above the sequences.

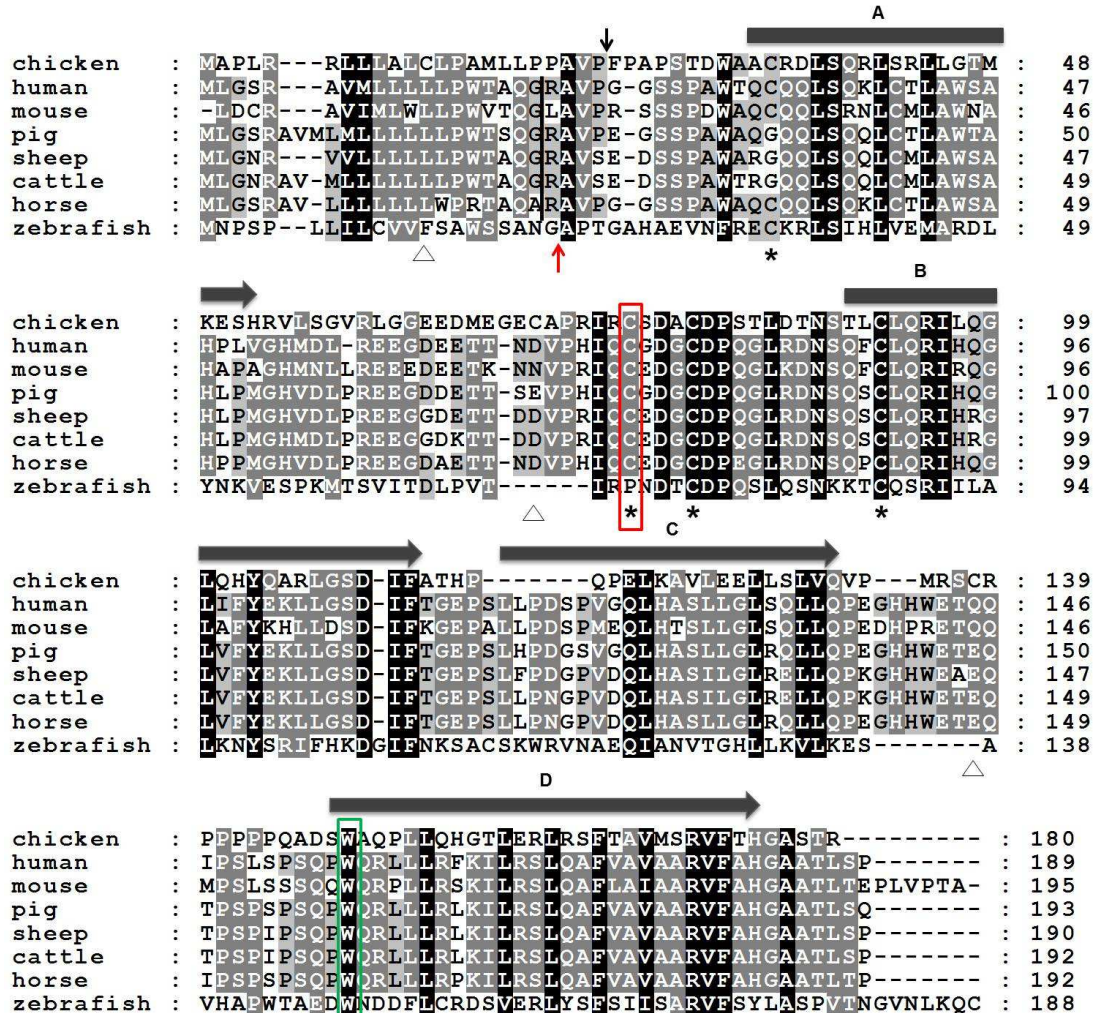


Figure 3.6. Comparison of the chIL-23 p19 aa sequence with mammalian and zebrafish IL-23 p19 aa sequences. Shaded areas represent conservation of aa similarity, the darker the shading the more conserved the residue is across the species. The vertical black line indicates the signal sequence cleavage site of mammalian IL-23 p19. The vertical red arrow is the predicted signal sequence cleavage site of zebrafish IL-23 p19. The vertical black arrow is the predicted signal sequence cleavage site of chIL-23 p19. Chicken-only cysteine residues are identified by a triangle. Conserved cysteine residues are identified by an asterisk. The red box highlights C76 (chIL-23 p19), the cysteine residue which interacts with p40 in mammals. The green box highlights W149 (chIL-23 p19), the tryptophan that engages with the IL-23R in mammals. Horizontal black arrows highlight the 4 α -helices, A, B, C & D, in human and mouse IL-23 p19.

3.3.3 Gene organisation of *chIL-23 p19*

Nucleotide BLAST analysis against the chicken genome (Galgal 4.0, Ensembl) shows that *chIL-23 p19* is encoded by four exons (Figure 3.7). In the genomic sequence, the intron splicing consensus (GT/AG) is conserved at the 5' and 3' ends of the introns. In the 3' UTR, 4 mRNA instability motifs (ATTTA) were present upstream of the polyA signal (AATAAA).

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63992 ATGGCCCCGCTCCGCCGCTCCTGCTCGCACTCTGCCTCCCGCGATGCTGCTGCCGCC 64051
64052 GCGGTCCCTTCCCGGCCCCGAGCACCGACTGGGCCGCTGCAGGGACCTCTCTCAGCGG 64111
64112 CTGTGCGCGGTGCTGGGGACAATGAAGGAGTCGCACCGCGTTCTGAGCGCCGGGACG 64171
64172 GGGTTTGGGGAGCGGGGAGACGAGGGATGCTATGGGAACGGGAGCACCGGGGGCACTC 64231
64232 AGGGAACCGGGGTGCTCCGGAACTGGAGATACTCAGGGAAGTGGAAATGCTCTGGGAA 64291
64292 CCGGGAGAACCGGGGATGCTCTCAGAACCGGGGAAATGGGGATGCTCGGGGAAGCAGAG 64351
64352 GTGCTCCAGGAAGTGGGAGTGCTCGGGGAAGTGGGAGAGCTGGGGATGCTCCTGGAACCG 64411
64412 GGGGTGTGGGACGCGGGGGTCTGGGGTACTGTGACTCTCCGGCTGCCTGGGGGAGC 64471
64472 CAAGGTGCTATGAGGTCCCTGTAACTCCCACTGTAGAGCGGGTCCGTCTGGGTGGAG 64531
64532 AGGAGGACATGGAGGGGAGTGTGCCCCCGCATCCGCTGCAGTGACGCCTGCGACCCCT 64591
64592 CCACGCTGGACACGAACAGCACGTACAGAGGGGGGTGGGGGGGAGTCTGAGGGGGCC 64651
64652 AGGGGGTATCCCTGGAGCCAAAGCCCTGGGGGTCTTGTGGGTGCGTGGATGCTCCGGG 64711
64712 ATGCATTGCGATGTCTGAAGGGTCTGGGTGGGGTCTGGGTGGGGTCTGGGGGTCT 64771
64772 CAGGGGTGCTGGGGTCCCAGAAGTGCTACGGGTGTTCCGGGGGGGAGGGGGGAAGGG 64831
64832 GTAGTGATGCTGGAGATGTTCTGGGGCATCTTGAAGCACCCAAGTGATGTGCTTGGG 64891
64892 GATGTCAGGGTAGCCTGGGGTCTCGGGGCTGTTGGGATCCAGAAGATCCCCAGGGGTG 64951
64952 CTTTGGGGACATGCTCAGGGGTCCAGCATCTTCATGCCCCCACCACCCACCCCTCTCT 65011
65012 GCCTGCAGCGGATCCTGCAGGGGTGCAGCACTACCAGGCCAGGCTGGGCTCCGACATCT 65071
65072 TCGCCACACACCCACAGCCGAGCTGAAGGCGGTGCTGGAGGAGCTGCTGAGCCTCGTGC 65131
65132 AGGTGAGCTGACCCCAACCCCAACCCCAACCCCAACCCGATCTTAACATCAACCCCAAC 65191
65192 CCCAACCCGAGCCCTCTGACCCCTCTCTCCCTGAGTCCCCATGCGTTCCTGCCGGCC 65251
65252 CCCCCCGCCCCCAAGCAGACTCCTGGGCTCAGCCACTGCTGCAGCACGGCACACTCGA 65311
65312 ACGGCTCCGCTCCTTACCGCCGTATGAGCCGCTTTCACCCACGGCGCCAGCACCCG 65371
65372 CTGACCGCCCCGACCTCCCCCCCCCTCCCCCGTTATTTATTTCTTTATTTATGGCCGCG 65431
65432 CTCCCTATTTATACGGGTATTTATGGGTTTCTAATAAAGGGCTGGGCAACGAAGAGCTG 65491
65492 CTCTCTGCTACAACGCCGCACAGGGCTGCC 65523

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Figure 3.7. Compiled genome sequence of the chicken IL-23 p19 gene. Exons are in red and introns are in black. Intron splice sites are boxed. Instability motifs (ATTTA) and the polyadenylation signal (AATAAA) are underlined.

3.3.4 *chIL-23 flexi-construct design*

A chIL-23 flexi-construct (Figure 3.1), based on the mammalian IL-12 flexi-construct (Lieschke *et al.*, 1997) and the chIL-12 flexi-construct (Degen *et al.*, 2004) was designed for recombinant protein expression and subsequent comparison of the biological activity of chIL-23 with mammalian IL-23. The chIL-23 flexi-construct is a single chain heterodimeric construct in which the mature proteins of the chicken p40 and p19 subunits are linked to each other by an in-frame glycine-serine-linker (flexi-linker) (Figure 3.8). This flexi-linker ensures both subunits are expressed together. The chIL-23 flexi-construct contains the mouse CD8 α signal peptide sequence to direct the recombinant protein for secretion from the transfected cell. An N-terminal V5HIS6 tag is included for purification of the recombinant protein. In addition, a precision protease site after the V5HIS6 tag allows easy cleavage, should the tag interfere with expressed protein function.

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ATGGCCTCACCGTTGACCCGCTTCTGTGCTGAACCTGCTGCTGCTGGGTGAGTCGATT
M A S P L T R F L S L N L L L L G E S I
ATCTGGGGAGTGGAGAAGGTAAGCCTATCCCTAACCTCTCCTCGGTCTCGATTCTACG
I L G S G E G K P I P N P L L G L D S T
CGTACCGGTCATCATCATCATCATCTGGAAGTTCGTTCAGGGGCCGCCCTTCTG
R T G H H H H H H L E V L F Q G P A L L
GAAGCACAGTGGAACTTAGAGAGAATGTGTATGTCATAGAATCTGAGTGGAAACGATGAG
E A Q W K L R E N V Y V I E S E W N D E
ACACCAGCTAAAAAGTGAAGCTCACCTGTGACACATCTGATGAAGCACTGCCAGTTTAC
T P A K K V K L T C D T S D E A L P V Y
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W K K G T E L K G T G K T L T T E V K E
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F P D A G N Y T C L S A K T H E I I S Y
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S F F L I T K V D S N G Q M I R S I L K
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S Y K E P S K T F L K C E A K N Y S G I
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F T C S W M T E N E S P S V K F T I R S
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L K G S Q G D V T C S S P V A R T D K S
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V T E Y T A Q C Q K E N Y C P F A E E H
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Q P T E M F L E V I D E V E Y E N Y T S
AGCTTCTTCATCAGAGATATCATAAAGCCAGACCCACCTCAATGTCTAGTATGCAAGCACA
S F F I R D I I K P D P P Q C Q Y A S T
AATGGAAGTGTGACCTGGACATATCCCAAGACCTGGAGCACACCGAAGTCTACTTCCCT
N G T V T W T Y P K T W S T P K S Y F P
TTGACTTTTCAGGGTCAAAGTTGAAAGCACAAAGAAATACAAAAGCAAGGTTTATGATGCT
L T F R V K V E S T K K Y K S K V Y D A
GATGAGCAGTCTATTTCAGATTCCAAAGACTGGGCCAAAAGACAAGATCTGTGTGCGAGCC
D E Q S I Q I P K T G P K D K I S V Q A
AGGGATCGCTATTACAACCTCATCTGGAGTGAGTGGTCCACGCTTTGCAGAGGCTCTGGC
R D R Y Y N S S W S E W S T L C R G S G
TCTTCTCGTGGCGGCTCTGGCTCTGGCGGCTCTGGCGGCGGCGCTCTAAACTGCCCGCG
S S R G G S G S G G S G G G S K L P A
GTCCCTTCCCGGCCCGGAGCACCGACTGGGCGGCTGTCAGGGACCTCTCTCAGCGGCTG
V P F P A P S T D W A A C R D L S Q R L
TCGCGGCTGCTGGGGACAATGAAGGAGTCGCACCGCGTTCTGAGCGGGGTCCGTCTGGGT
S R L L G T M K E S H R V L S G V R L G
GGAGAGGAGGACATGGAGGGGGAGTGTGCCCCCGCATCCGCTGCAGCGACGCTGCGAC
G E E D M E G E C A P R I R C S D A C D
CCCTCCACGCTGGACACGAACAGCACGCTCTGCCTGCAGCGGATCCTGCAGGGGCTGCAG
P S T L D T N S T L C L Q R I L Q G L Q
CACTACCAGGCCAGGCTGGGCTCCGACATCTTCGCCACACACCCACAGCCGAGCTGAAG
H Y Q A R L G S D I F A T H P Q P E L K
GCGGTGCTGGAGGAGCTGCTGAGCCTCGTGCAGGTCCCCATGCGTTCTTCCGCGCCCCC
A V L E E L L S L V Q V P M R S C R P P
CCGCCCCCCCCAAGCAGACTCCTGGGCTCAGCCACTGCTGCAGCACGGCACACTCGAACGG
P P P Q A D S W A Q P L L Q H G T L E R
CTCCGCTCCTTCAACGCCGTCATGAGCCGCGTCTTACCCACGGCGCCAGCACCCGCTGA
L R S F T A V M S R V F T H G A S T R -

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Figure 3.8. chIL-23 flexi-construct nt and aa sequence alignment. The mouse CD8 α signal peptide is shown in italics. The V5HIS6 tag is indicated in red. The precision protease site is highlighted in bold. The p40 mature protein sequence follows the precision protease site in blue. The flexi-linker sequence is underlined with the p19 mature protein sequence, followed by a stop codon, in green.

3.3.5 Bioactivity of recombinant chIL-23

In mammals, IL-23 stimulation of memory T cells drives expression of IL-17A (Aggarwal *et al.*, 2003). The chIL-23 flexi-construct was used to transiently express recombinant protein in COS-7 cells and rchIL-23 was also expressed and purified by Dundee Cell Products. Using both ex-COS and purified rchIL-23, a bioassay was developed and optimised to demonstrate bioactivity of rchIL-23 (section 2.9.2). The bioassay was based on the previously described IL-23 bioassay in mammals (Aggarwal *et al.*, 2003), measuring the expression of chIL-17A mRNA by splenocytes in response to chIL-23. In mammals, IL-17A and IL-17F mRNA and protein expression were measured over time by RT-qPCR and enzyme-linked immunosorbent assay (ELISA) respectively. The measurement of Th17 cytokine protein expression is not possible in the chicken as Abs have not been raised to all of the cytokines. Therefore, mRNA expression of Th17 effector cytokine transcripts was measured by RT-qPCR (TaqMan®).

Chicken splenocytes, when stimulated with purified rchIL-23, with or without the presence of rchIL-2, initially up-regulated chIL-17A mRNA expression compared to levels in unstimulated controls, with chIL-17A mRNA expression then decreasing with decreasing concentrations of IL-23-stimulation (Figure 3.9). This quadratic decline of chIL-17A mRNA levels was enhanced in the presence of rchIL-2 ($p = 0.007$).

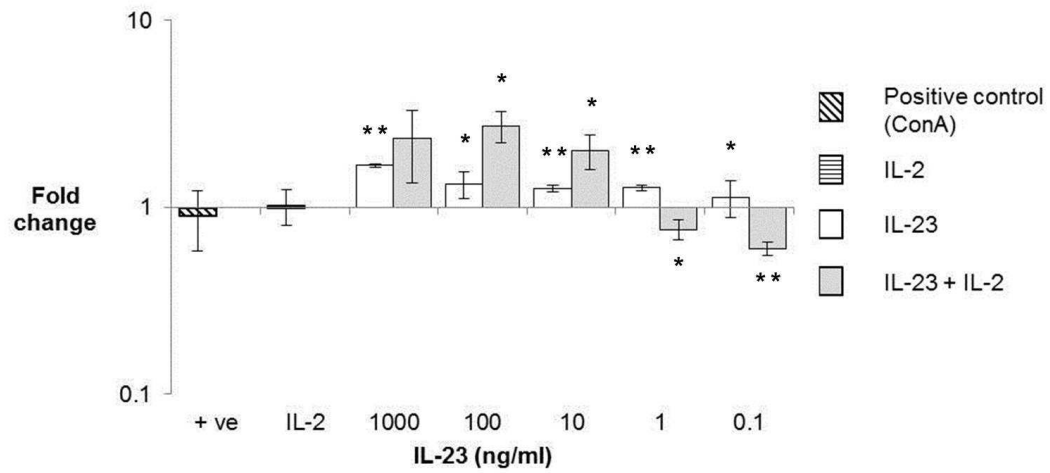


Figure 3.9. Chicken IL-17A mRNA levels in purified rchIL-23-stimulated splenocytes. Cells were stimulated for 1 d with serial dilutions of purified rchIL-23 or purified rchIL-23 and ex-COS rchIL-2. Data are expressed as fold change compared to levels in age-matched unstimulated controls \pm SEM. A mixed effect model was used to determine if there was a relationship between mRNA levels over time. A standard one-sample *t* test was performed to determine if cytokine levels were significantly up or down-regulated relative to a fold change of one as indicated by a single asterisk ($p < 0.05$) or double asterisk ($p < 0.01$). The positive control (+ ve) was ConA-stimulated splenocytes (1 μ g/ml) and ex-COS rchIL-2 was used at a 1/500 dilution. Medium alone was used on the unstimulated cells. Assays were carried out in triplicate and each value is the mean of 3 birds.

As chIL-17A mRNA levels induced by IL-23-stimulation were not that high, mRNA expression of the other Th17 effector cytokines, chIL-17F, chIL-21 and chIL-22, were investigated.

Following stimulation of splenocytes with purified rchIL-23 for 6 h, 1, 2, 3, 4 and 6 d, chIL-17F mRNA levels were significantly up-regulated at all time-points ($p < 0.026$) compared to levels in unstimulated controls (Figure 3.10).

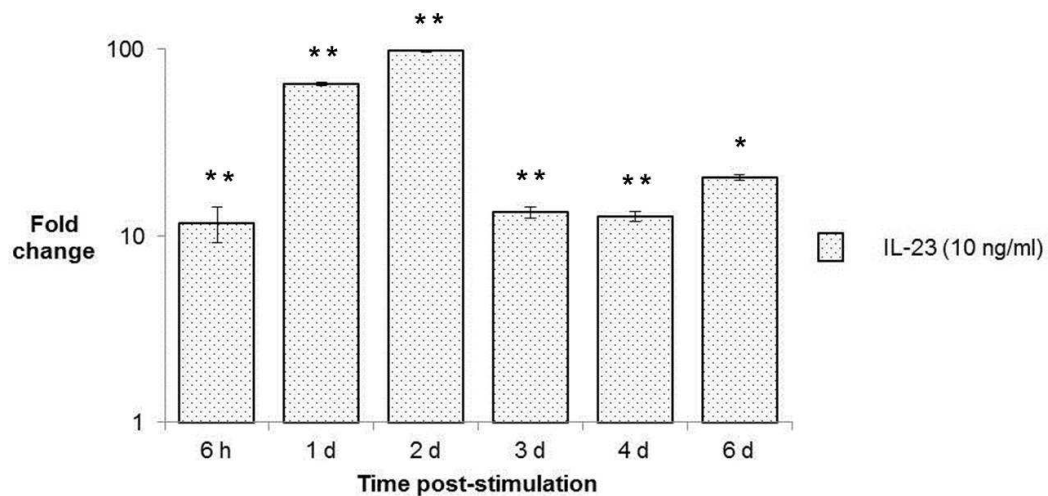


Figure 3.10. Chicken IL-17F mRNA levels in splenocytes stimulated with purified rchIL-23 (10 ng/ml). Data are expressed as fold change compared to levels in age-matched unstimulated controls \pm SEM. A mixed effect model was used to determine if there was a relationship between mRNA levels over time. A standard one-sample t test was performed to determine if cytokine levels were significantly up or down-regulated relative to a fold change of one as indicated by a single asterisk ($p < 0.05$) or double asterisk ($p < 0.01$). Medium alone was used on the unstimulated cells. Assays were carried out in triplicate and each value is the mean of 6 birds.

Chicken IL-17F mRNA levels increased initially over time, before declining at 3-6 d of stimulation. The quadratic decline of chIL-17F mRNA levels over time was demonstrated at 1000 and 10 ng/ml of IL-23-stimulation ($p < 0.045$). However, at concentrations of 100 and 1 ng/ml, the decline of chIL-17F mRNA levels over time was linear ($p < 0.006$).

Figure 3.11 shows the effect of titrating purified rchIL-23 on chIL-17F mRNA expression from splenocytes at 2 d post-stimulation. Concentrations of rchIL-23 from 1-1000 ng/ml significantly up-regulated chIL-17F mRNA levels ($p < 0.014$). A dose-dependent effect was seen, with a typical prozoan effect, with highest concentrations giving less than maximal stimulation and effects titrating out, with increasing dilution, to background levels. This quadratic decline ($p < 0.015$) in chIL-17F mRNA expression was seen from 6 h to 4 d post-stimulation. By 6 d post-stimulation the decline in chIL-17F mRNA levels was linear ($p < 0.001$).

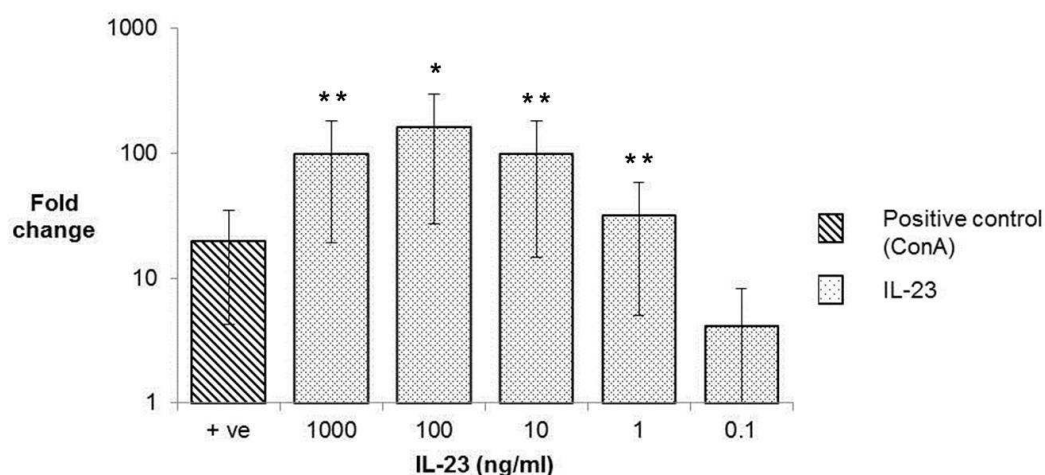


Figure 3.11. Chicken IL-17F mRNA levels in purified rchIL-23-stimulated splenocytes. Data are expressed as fold change compared to levels in unstimulated controls \pm SEM. A mixed effect model was used to determine if there was a relationship between mRNA levels over time. A standard one-sample t test was performed to determine if cytokine levels were significantly up or down-regulated relative to a fold change of one as indicated by a single asterisk ($p < 0.05$) or double asterisk ($p < 0.01$). Cells were stimulated for 2 d. The positive control (+ ve) was ConA-stimulated splenocytes (1 μ g/ml). Medium alone was used on the unstimulated cells. Assays were carried out in triplicate and each value is the mean of 6 birds.

The original mammalian IL-23 bioassay investigated the effect of IL-23 and IL-2 co-stimulation on expression of IL-17A and IL-17F (Aggarwal *et al.*, 2003). IL-2 drives the proliferation of T cells (Smith, 1984). However, Laurence *et al.* (2007) showed IL-2 could inhibit the differentiation of Th17 cells. The cytokines which drive or

inhibit Th17 immune responses in the chicken have not been studied to date, therefore, for completeness, the effect of rchIL-2 (ex-COS) stimulation on chicken splenocytes was studied. Purified rchIL-23 alone (1-1000 ng/ml), and in combination with rchIL-2 (ex-COS), significantly up-regulated chIL-17F mRNA levels in splenocytes ($p < 0.002$) (Figure 3.12). Purified rchIL-23 in combination with rchIL-2 (ex-COS) significantly increased chIL-17F mRNA expression compared to stimulation with IL-23 alone between 6 h and 2 d post-stimulation ($p < 0.001$) but not after 3-6 d post-stimulation.

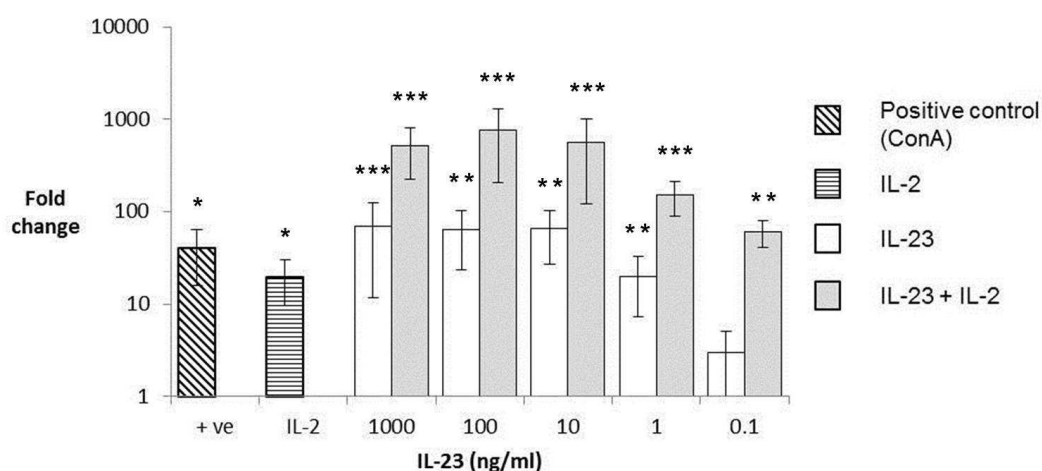


Figure 3.12. Chicken IL-17F mRNA levels in purified rchIL-23-stimulated splenocytes. Cells were stimulated for 1 d with serial dilutions of purified rchIL-23 or purified rchIL-23 and ex-COS rchIL-2. Data are expressed as fold change compared to levels in age-matched unstimulated controls \pm SEM. A mixed effect model was used to determine if there was a relationship between mRNA levels over time. A standard one-sample t test was performed to determine if cytokine levels were significantly up or down-regulated relative to a fold change of one as indicated by a single asterisk ($p < 0.05$), double asterisk ($p < 0.01$) or triple asterisk ($p < 0.001$). The positive control (+ ve) was ConA-stimulated splenocytes (1 μ g/ml) and ex-COS rchIL-2 was used at a 1/500 dilution. Medium alone was used on the unstimulated cells. Assays were carried out in triplicate and each value is the mean of 6 birds.

Chicken IL-17A and chIL-17F mRNA expression was up-regulated in splenocytes after stimulation with purified rchIL-23. Levels of chIL-21 and chIL-22 mRNA from splenocytes stimulated with purified rchIL-23 were also investigated. Chicken IL-21 mRNA was not up- or down-regulated following rchIL-23 stimulation compared to levels in unstimulated controls (Figure 3.13). However, the addition of rchIL-2 (ex-COS) significantly increased mRNA levels of chIL-21 compared to rchIL-23-stimulation alone ($p = 0.016$).

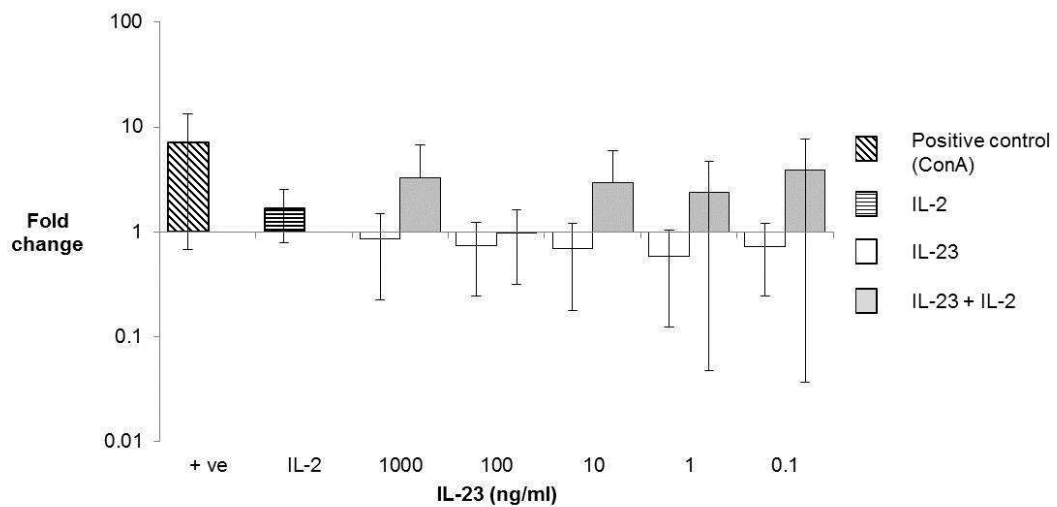


Figure 3.13. Chicken IL-21 mRNA levels in purified rchIL-23-stimulated splenocytes. Cells were stimulated for 1 d with serial dilutions of purified rchIL-23 or purified rchIL-23 and ex-COS rchIL-2. Data are expressed as fold change compared to levels in age-matched unstimulated controls \pm SEM. A mixed effect model was used to determine if there was a relationship between mRNA levels over time. A standard one-sample t test was performed to determine if cytokine levels were significantly up or down-regulated relative to a fold change of one. The positive control (+ ve) was ConA-stimulated splenocytes (1 μ g/ml) and ex-COS rchIL-2 was used at a 1/500 dilution. Medium alone was used on the unstimulated cells. Assays were carried out in triplicate and each value is the mean of 3 birds.

Lastly, chIL-22 mRNA levels were measured in splenocytes stimulated with purified rchIL-23. Chicken IL-22 mRNA expression was significantly up-regulated by rchIL-23 stimulation compared to unstimulated controls at all concentrations of purified rchIL-23-stimulation ($p < 0.027$) (Figure 3.14). Recombinant chIL-23 also had a prozone effect on chIL-22 mRNA levels in splenocytes. The highest chIL-22 mRNA levels were measured at 100 ng/ml of rchIL-23-stimulation, and chIL-22 mRNA levels then declined as rchIL-23 was titrated. This quadratic relationship was seen from 6 h to 4 d ($p < 0.007$). By 6 d post-stimulation, the decline in chIL-22 mRNA levels was linear ($p < 0.001$).

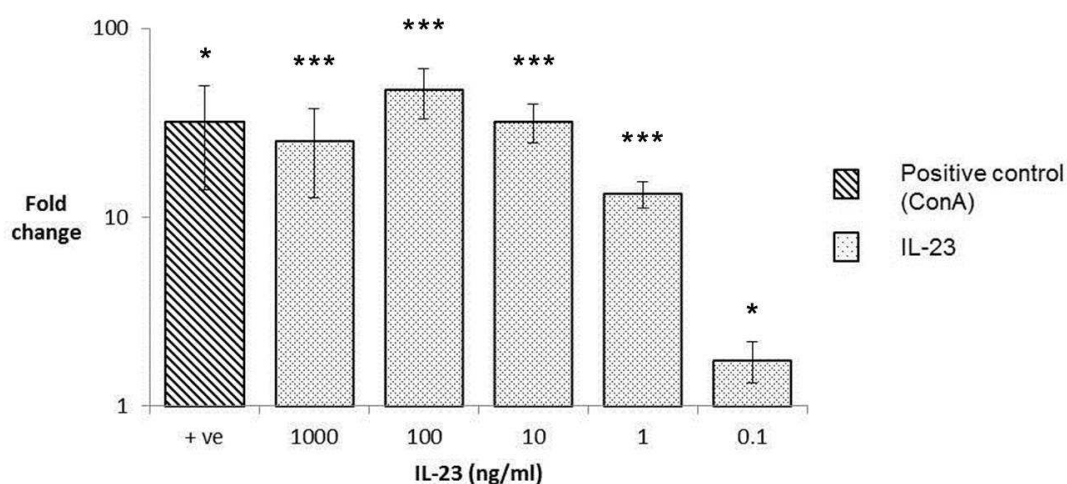


Figure 3.14. Chicken IL-22 mRNA levels in purified rchIL-23-stimulated splenocytes. Data are expressed as fold change compared to levels in age-matched unstimulated controls \pm SEM. A mixed effect model was used to determine if there was a relationship between mRNA levels over time. A standard one-sample t test was performed to determine if cytokine levels were significantly up or down-regulated relative to a fold change of one as indicated by a single asterisk ($p < 0.05$), double asterisk ($p < 0.01$) or triple asterisk ($p < 0.001$). Cells were stimulated for 6 h. The positive control (+ ve) was ConA-stimulated splenocytes (1 μ g/ml). Medium alone was used on the unstimulated cells. Assays were carried out in triplicate and each value is the mean of 6 birds.

In summary, stimulation of chicken splenocytes with purified rchIL-23 has a prozone effect on chIL-17A, chIL-17F and chIL-22 mRNA expression. In contrast, chIL-21 mRNA expression in splenocytes was not changed by stimulation with purified

rchIL-23. The addition of rchIL-2 (ex-COS) generally enhanced mRNA expression of chIL-17A, chIL-17F and chIL-21. These data demonstrate purified rchIL-23 is bioactive.

3.3.6 Recombinant chIL-23-stimulated dendritic cells do not express chIL-12 p35

IL-23-stimulated murine splenic DCs express IL-12 p70 and IFN- γ (Belladonna *et al.*, 2002). It is currently not possible to differentiate splenic DCs *in vitro* in the chicken, therefore, using an established method (Wu *et al.*, 2010), chicken BMDCs were differentiated and stimulated with a series of dilutions of rchIL-23 (ex-COS) (sections 2.7.4 and 2.7.5) and mRNA expression of chIL-12 p35 measured using RT-qPCR (TaqMan®).

Chicken BMDCs stimulated with rchIL-23 (ex-COS) did not up-regulate mRNA levels of chIL-12 p35 (Figure 3.15).

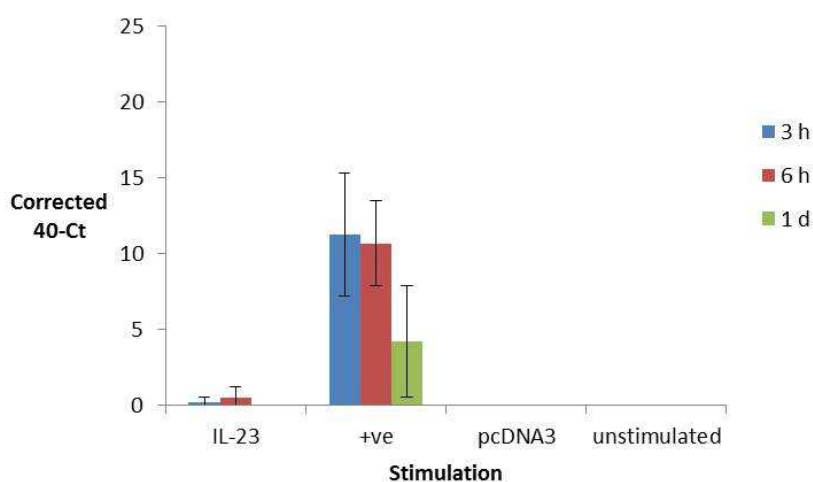


Figure 3.15. Chicken IL-12 p35 mRNA expression levels in BMDCs stimulated with rchIL-23 (ex-COS). Data are expressed as corrected 40-Ct values \pm SEM. BMDCs were stimulated with rchIL-23 (ex-COS) diluted 1/100, LPS 200 ng/ml (+ve control), pcDNA3 (ex-COS) diluted 1/100 and medium alone (-ve control) for 3 h (blue bars), 6 h (red bars) and 24 h (green bars) respectively. Assays were carried out in triplicate and each value is the mean of 5 birds.

After differentiating, the BMDCs were stimulated for 3, 6 and 24 h with ex-COS rchIL-23 diluted 1/100 and 1/250. Figure 3.15 shows results from BMDCs stimulated with 1/100 ex-COS rchIL-23. Similar results were seen with a 1/250 dilution of rchIL-23 (data not shown). Altering the concentration of rchIL-23 used to stimulate BMDCs had no effect on chIL-12 p35 mRNA expression.

3.3.7 *Chicken anti-IL-12 p40 monoclonal antibodies bind recombinant chIL-23 protein*

IL-23 is composed of the subunits p19 and p40, the latter of which it shares with IL-12 (Oppmann *et al.*, 2000). Monoclonal antibodies specific for chIL-12 have been generated and characterised in this laboratory (Balu *et al.*, 2011). Dot-blot analysis was used to determine if mouse anti-chicken IL-12 p40 mAbs recognise purified rchIL-23.

Three mouse anti-chIL-12 p40 mAbs (AV134, AV135 and AV136), which recognise rchIL-12 p40 and rchIL-12 p70, were used against a dilution series of purified rchIL-23. All three mAbs detected purified rchIL-23 (Figure 3.16). Recombinant chIL-12 p40 and rchIL-7 (both ex-COS) were used as positive and negative controls respectively. Monoclonal Ab AV134 detected purified rchIL-23 at concentrations of 12 and 60 µg/ml (1/25 and 1/5 dilutions respectively) but not diluted 1/125 at a concentration of 2.4 µg/ml. Monoclonal Abs AV135 and AV136 detected all concentrations of purified rchIL-23. All three mouse anti-chIL-12 p40 mAbs detected rchIL-12 p40 (ex-COS) but not the negative control, rchIL-7 (ex-COS). The experiment was repeated three times.

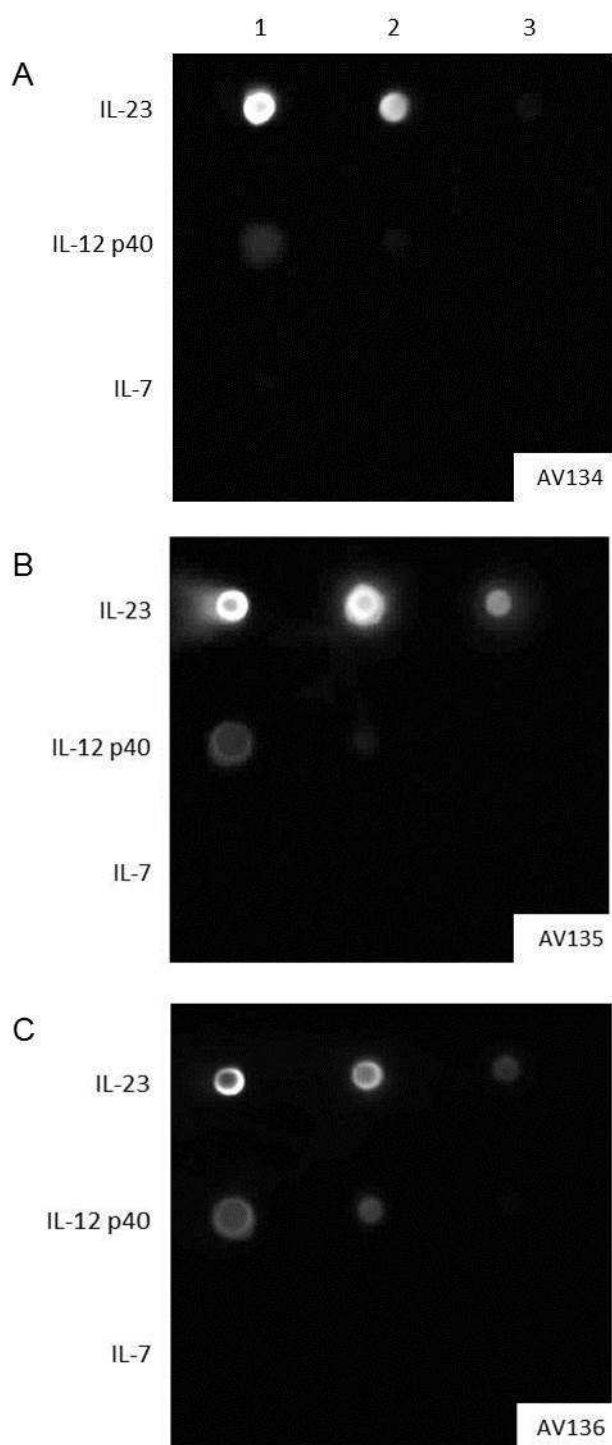


Figure 3.16. Dot-blot analysis demonstrating specificity of mouse anti-chicken IL-12 p40 monoclonal antibodies (A) AV134, (B) AV135 and (C) AV136 against a series of dilutions of purified rchIL-23, rchIL-12p40 (ex-COS) and rchIL-7 (ex-COS). Purified rchIL-23 was diluted (1) 1:5 (60 µg/ml), (2) 1:25 (12 µg/ml) and (3) 1:125 (2.4 µg/ml). Recombinant chIL-12 p40 and rchIL-7 were used (1) neat, and at (2) 1:10 and (3) 1:100 dilutions.

3.3.8 Expression of *chIL-23 mRNA* in tissues

Having confirmed the chIL-23 p19 nt sequence, it was now possible to measure mRNA levels of chIL-23 in various tissues, stimulated cells and during infection of the chicken. Attempts to design TaqMan® primers and probe for use in a RT-qPCR assay to measure mRNA expression of chIL-23 p19 were unsuccessful. Further attempts to design SYBR® Green primers by eye and to optimise a SYBR® Green RT-qPCR assay, including 1-step and 2-step systems, were also unsuccessful. The high GC content of the chIL-23 p19 nt sequence proved insurmountable. Therefore, a custom assay was designed by Applied Biosystems (Table 2.4).

As shown in Figure 3.17, chIL-23 p19 mRNA is widely expressed in all immune and non-immune tissues investigated at moderate levels. The highest mRNA levels in the non-lymphoid tissues were in the kidney and in lymphoid tissues in the spleen, with very similar levels seen in the thymus, Meckel's diverticulum and bone marrow.

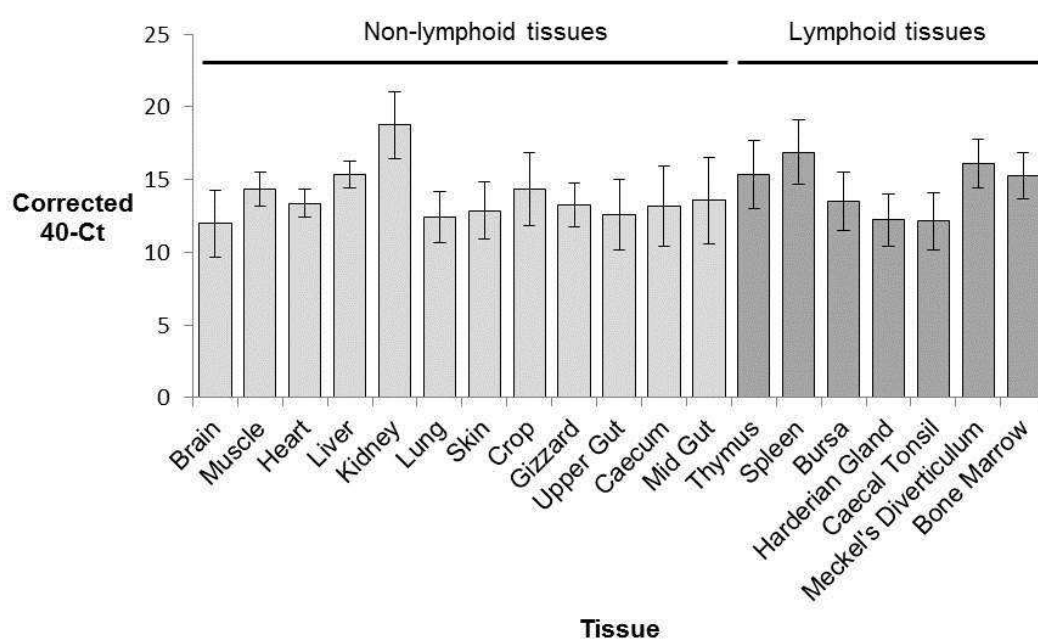


Figure 3.17. Expression patterns of chIL-23 p19 mRNA in tissues as measured by RT-qPCR. Results are expressed as corrected 40-Ct values \pm SEM. Assays were carried out in triplicate and each value is the mean of 3 birds.

3.3.9 Expression of chIL-23 mRNA in stimulated splenocytes, thymocytes and bursal cells across a time course

Chicken IL-23 p19 mRNA levels were measured in stimulated lymphoid cells and compared to levels in unstimulated controls. Splenocytes were stimulated with ConA, thymocytes with PHA and bursal cells with PMA and ionomycin over a time course of 2-24 h. Chicken IL-23 p19 mRNA levels were significantly down-regulated at 18 h ($p = 0.042$) post-stimulation in ConA-stimulated splenocytes (Figure 3.18 (A)). Expression of chIL-23 p19 mRNA compared to unstimulated controls decreased over time in a linear relationship ($p = 0.004$). Chicken IL-23 p19 mRNA levels in stimulated thymocytes was up-regulated at 2 h post-stimulation but there was no relationship in chIL-23 p19 mRNA levels in stimulated thymocytes over time (Figure 3.18 (B)). Expression of chIL-23 p19 mRNA in stimulated bursal cells significantly decreased over time in a linear relationship ($p < 0.009$) (Figure 3.18 (C)). Chicken IL-23 p19 mRNA levels were significantly down-regulated at 12 h ($p = 0.049$) post-stimulation.

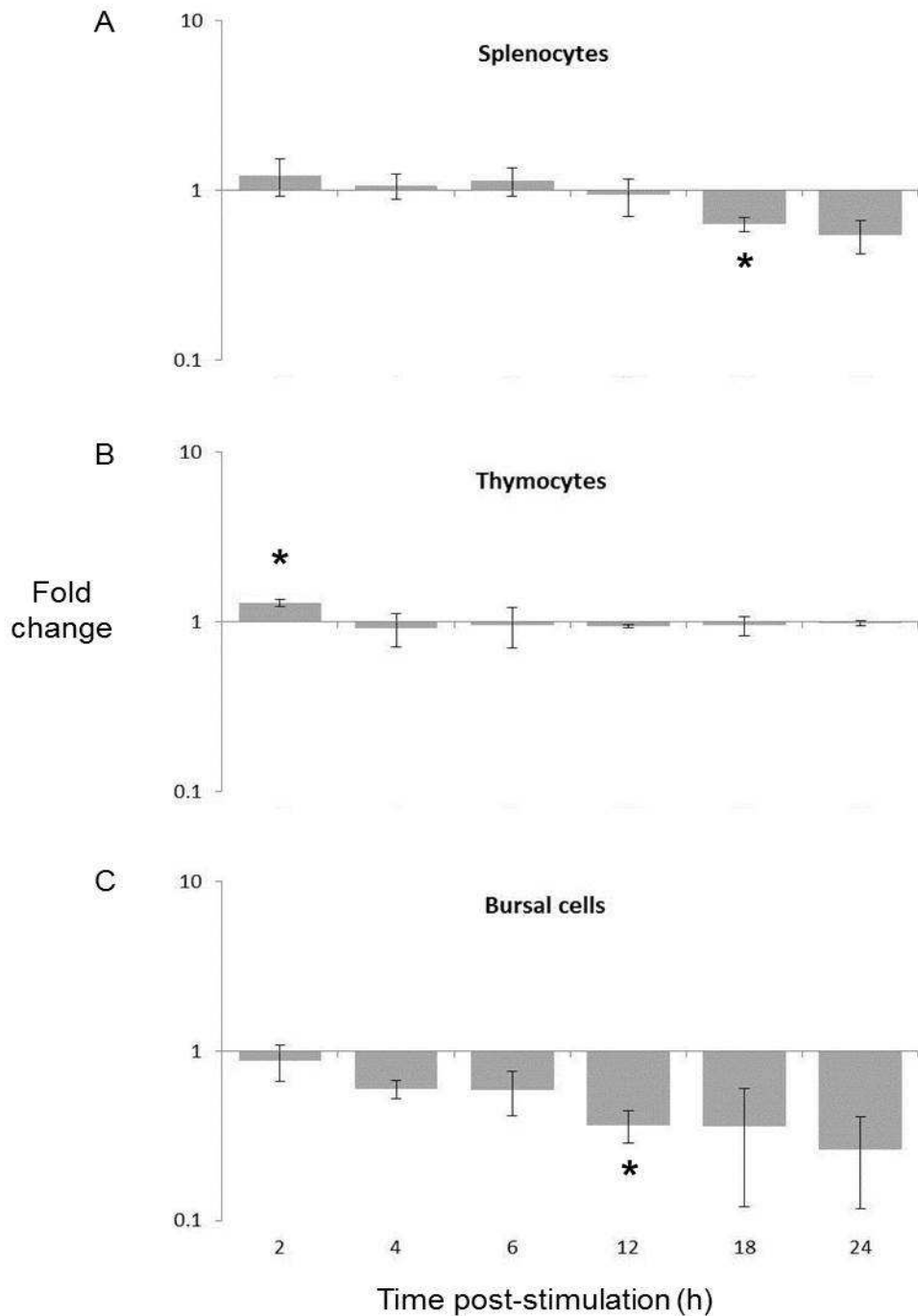


Figure 3.18. Expression patterns of chIL-23 p19 mRNA in stimulated (A) splenocytes, (B) thymocytes and (C) bursal cells. Data are expressed as fold change compared to levels in age-matched unstimulated controls \pm SEM. A linear mixed effect model was used to determine if there was a relationship between mRNA levels over time. A standard one-sample t test was performed to determine if cytokine levels were significantly up or down-regulated relative to a fold change of one as indicated by a single asterisk ($p < 0.05$). Chicken splenocytes, thymocytes and bursal cells were stimulated with ConA, PHA and PMA and ionomycin respectively. Assays were carried out in triplicate and each value is the mean of 3 birds.

3.4 Discussion

Interleukin-23 was first identified in mammals nearly 15 years ago through its similarity to members of the IL-6 cytokine family (Oppmann *et al.*, 2000). Detectable levels of the p19 subunit combined with the p40 subunit of IL-12, forming a biologically active cytokine, were shown to be secreted by activated DCs. Uniquely at the time, IL-23 induced strong proliferation of mouse memory T cells. Subsequently, Aggarwal *et al.* (2003) showed IL-23 promoted differentiation of a new subset of CD4⁺ T cells that produce IL-17A. More than 10 years on, the extensive role of IL-23 in driving both the innate and adaptive immune responses in mammals is still being elucidated. This Chapter describes the identification and initial characterisation of IL-23 in the chicken.

The initial aim of this Chapter was to identify the chicken IL-23 p19 gene. The chicken IL-12/IL-23 p40 subunit was identified in the chicken in 2003 (Balu and Kaiser, 2003) and a functional recombinant chIL-12 protein expressed in 2004 (Degen *et al.*) but version 4 of the chicken genome (Galgal 4.0, Ensembl) still does not have an annotated chIL-23 p19 gene. Because of low sequence homology to mammalian cytokines, classical molecular approaches to characterise avian cytokine homologues are not usually successful. The protein coding sequence (CDS) for chIL-23 p19 was identified using *in silico* and *in vitro* methods. Several unsuccessful attempts were made to amplify the 5' and 3' ends of the chIL-23 p19 cDNA by 5' and 3' RACE-PCR to elucidate its full-length sequence. Subsequently, a predicted nt sequence for full-length chIL-23 p19 (LOC100858964) was located in the NCBI nt database. Using primers based on this predicted sequence, the full-length nt sequence of chIL-23 p19 was cloned by PCR. On sequencing the PCR product, the predicted

nt sequence (LOC100858964) was found to be incorrectly predicted. The precise genome location is yet to be determined as the predicted chIL-23 p19 nt sequence was identified in an unmapped area of the chicken genome. Therefore, it is not possible to say if the chIL-23 p19 gene demonstrates conserved synteny with mammals. However, the gene organisation of chIL-23 p19 has 4 exons and 3 introns, and is similar to that of human and mouse IL-23 p19.

Following identification of the chIL-23 p19 gene, the aa sequence was compared to its mammalian counterparts. Chicken IL-23 p19 is similar in aa sequence length to mammalian IL-23 p19 proteins and has a moderately high aa identity, for an avian cytokine, of 35.5% compared to mammalian IL-23 p19 (Kaiser *et al.*, 2005). The aa identity is lower than that of chIL-12 p40, which has an aa identity of 46% and 41% respectively with huIL-12 p40 and mIL-12 p40 (Balu and Kaiser, 2003). The predicted signal sequence cleavage site in chIL-23 p19 is similar to the known signal sequence cleavage sites in mammalian IL-23s. Human IL-23 is a heterodimeric four-alpha-helical bundle cytokine, expressed as a disulphide-linked complex of the helical cytokine p19 and its binding protein p40 (Lupardus and Garcia, 2008). Four-alpha helical bundle cytokines, such as IL-23, share a common structural motif made up of a central core of four α -helices (A, B, C and D), arranged in an up-up-down-down helical topology (Sprang and Bazan, 1993). It is clear from the aa alignment that there is good homology and conservation of heptad repeat structures between human and chicken aa sequences within the regions where the α -helices are predicted to be, suggesting the α -helical structures are the same in the chicken. The cysteine residues important for disulphide bond formation within the huIL-23 p19 subunit (Oppmann *et al.*, 2000) are conserved in the chicken and will likely form disulphide

bonds. Also, the cysteine residue which forms a disulphide bond with mammalian p40, and the tryptophan which interacts with the IL-23R in mammals (Lupardus and Garcia, 2008), are both conserved in the chicken, suggesting a likely conservation of function between mammalian and avian IL-23.

To investigate the biological function of chIL-23, a chIL-23 flexi-construct was designed for recombinant protein expression. Similar to mammalian IL-12, the two subunits, p35 and p40, of chicken IL-12 need to heterodimerise to exert bioactivity (Degen *et al.*, 2004). Therefore, it was assumed that chIL-23 would be similar to mammalian IL-23 and require expression of both subunits in the same cell for the recombinant protein to be biologically active. To this end, the design of the chIL-23 flexi-construct ensures the p40 and p19 subunits are expressed simultaneously in the same cell at the same level. Almost all of the key molecules in the immune system are glycosylated (Shirouzono *et al.*, 2012). Therefore, to compare biological activity of chIL-23 with mammalian IL-23, mammalian expression systems were used to express rchIL-23 to allow for post-translational modifications and hopefully generate a functional protein. The chIL-23 flexi-construct also contains a mouse CD8 α signal peptide to direct recombinant protein secretion from the cell.

To establish the biological function of rchIL-23, we sought to replicate the initial bioassay used in mice to investigate the biological function of IL-23 (Aggarwal *et al.*, 2003). Following the discovery of mammalian IL-23, in experiments using knockout mice deficient in either the p19 or p40 subunits, its involvement in the autoimmune disease MS was elucidated in the mouse model, EAE (Cua *et al.*, 2003). An IL-23 knockout model in the chicken to establish chIL-23 function, whilst potentially possible in the not-to-distant future, is unavailable in the chicken at

present. We therefore sought to generate bioactive rchIL-23 as a useful tool to begin to elucidate the functions of chIL-23 *in vitro* and *in vivo*.

In mammals, IL-23 drives expression of IL-17A and IL-17F from splenocytes (Aggarwal *et al.*, 2003). Chicken splenocytes were cultured in the presence of various concentrations of purified rchIL-23 alone and in combination with rchIL-2 for up to 6 d. Measurement of all Th17 cytokines at the protein level is not yet possible in the chicken so mRNA levels of chIL-17A, chIL-17F, chIL-21 and chIL-22 were measured. Recombinant chIL-23-stimulated splenocytes up-regulated expression of chIL-17A, chIL-17F and chIL-22 mRNA levels compared to unstimulated controls but there was no effect on mRNA levels of chIL-21.

As mammalian IL-23 drives expression of IL-17A, this cytokine was chosen as the initial readout to determine if rchIL-23 is biologically active. Chicken IL-17A expression declined as rchIL-23-stimulation decreased, particularly in the presence of rchIL-2. However, the positive control (ConA) failed to increase chIL-17A mRNA expression and the expression of chIL-17A mRNA from rchIL-23-stimulated splenocytes was low (< 5 fold change), suggesting the data should be interpreted cautiously. Therefore, the mRNA levels of the other Th17 effector cytokines, chIL-17F, chIL-21 and chIL-22, were also measured in the rchIL-23-stimulated splenocytes, using ConA as a positive control. ConA successfully increased mRNA expression of these cytokines (also shown in Chapter 4) but highest cytokine mRNA expression varied between 2-18 h post-stimulation. This is discussed further in section 4.4.2 but it may be that chIL-17A expression requires higher concentrations of ConA stimulation than was used here and highest expression may be seen earlier than 24 h post-stimulation.

Purified rchIL-23-stimulation of splenocytes up-regulated chIL-17F mRNA expression from 6 h to 6 d compared to levels in unstimulated controls. Chicken IL-17F mRNA levels declined over time. A dose-dependent effect of rchIL-23-stimulation was also seen, with the highest concentration of rchIL-23 (1000 ng/ml) giving less than maximal chIL-17F mRNA levels and effects titrating out, with decreasing concentration of rchIL-23-stimulation. This prozone, or hook effect, is a common biological response whereby the maximum response is achieved at less than maximal stimulation levels.

Chicken IL-22 mRNA levels were also up-regulated in rchIL-23-stimulated splenocytes, in a similar pattern to chIL-17F, whereas rchIL-23-stimulation had no effect on chIL-21 mRNA expression. Expression of IL-21 and IL-22 was not investigated in the original murine IL-23 bioassay. However, as discussed before (section 1.3.2), IL-23 is critical for IL-22 expression in mammals, both *in vitro* and *in vivo* (Liang *et al.*, 2006; Zheng *et al.*, 2007). IL-21 expression in mammalian Th17 cells acts via an autocrine loop, further enhancing expression of IL-21 and the IL-23R, thus augmenting responsiveness to IL-23 (Nurieva *et al.*, 2007). It is not surprising then that rchIL-23-stimulation increases expression of chIL-22 but does not up-regulate chIL-21 mRNA in splenocytes.

In mammals, IL-2 supports proliferation and survival of T cells (reviewed by Smith, 1984) but, contrary to this, is a negative regulator of Th17 development (Laurence *et al.*, 2007). In the original IL-23 bioassay outlined by Aggarwal *et al.* (2003), and subsequently by Kokuho *et al.* (2012) to demonstrate bioactivity of swine IL-23, IL-2 and IL-23 stimulation increased expression of IL-17 in splenocytes. The results here are in line with the mammalian bioassay and indicate that the addition of rchIL-

2 has a synergistic effect on mRNA levels of chIL-17A and chIL-17F and also increases chIL-21 mRNA levels alone. It is possible that rchIL-23-stimulation of splenocytes drives expression of chIL-17A and chIL-17F from innate cells in a similar manner to IL-23-driven expression of IL-17A in ILC3s in mammals (Spits *et al.*, 2013; Takatori *et al.*, 2009). Further elucidation of the cell types in the chicken spleen expressing chIL-17A, chIL-17F and chIL-22 is required to answer this. In mammals, IL-23 drives expression of IFN- γ and GM-CSF in Th17 cells (El-Behi *et al.*, 2011; Ivanov *et al.*, 2006). It would be interesting to further expand the biological role of chIL-23 to investigate whether it can also drive expression of chIFN- γ and chGM-CSF. Bioactivity of rchIL-23 has been demonstrated by the up-regulation of expression of three signature Th17 cytokines chIL-17A, chIL-17F and chIL-22, similarly to mammalian IL-23s (Aggarwal *et al.*, 2003), confirming its functionality and its usefulness as a tool to investigate the role of chIL-23 and Th17 immune responses in the chicken.

A further measure of IL-23 bioactivity in mammals is the induction of IL-12 protein expression by splenic DCs in mice (Belladonna *et al.*, 2002). Therefore, in addition to activating splenocytes, the biological effect of rchIL-23 on BMDCs was investigated and compared to mammals. Isolation and differentiation of splenic DCs *in vitro* in the chicken is not currently possible. With limited scope for an alternative, the established method of BMDC differentiation was used (Wu *et al.*, 2010). Bone marrow-derived DCs were differentiated and then stimulated with rchIL-23, and the mRNA levels of chIL-12 p35 measured. Expression of chIL-12 p35 mRNA was not detected in the rchIL-23-stimulated BMDCs despite LPS-stimulated positive controls indicating the cells could be stimulated to express chIL-12 p35. This result contrasts

with original mammalian studies (Belladonna *et al.*, 2002) but is in line with a very recent study by Chognard *et al.* (2014) where by IL-23R expression was not detected on mice DCs, suggesting these cells do not respond to IL-23. Despite the contradictory nature of the mammalian studies, it is not surprising chicken BMDCs do not express chIL-12 when stimulated with rchIL-23 as BMDCs compared to splenic DCs *in vitro* have different biological activities (Garrigan *et al.*, 1996). If a method for isolating and differentiating splenic DCs in the chicken were available, investigating the biological effect of rchIL-23-stimulation on splenic DCs would perhaps shed more light on the similarities and differences of mammalian IL-23 and chIL-23.

As discussed before, IL-23 and IL-12 share the p40 subunit. Monoclonal Abs developed by this group (Balu *et al.*, 2011) which recognise chIL-12 have been characterised. Specifically, the mAbs recognise the p40 subunit of chIL-12 and, therefore, should recognise the p40 subunit of chIL-23. To further investigate this, three anti-chicken IL-12 p40 mAbs, AV134, AV135 and AV136 were used in a dot-blot analysis to determine if they recognise purified rchIL-23. All three mAbs detect purified rchIL-23. It is now no longer possible to use the chIL-12 p40 mAbs for definitive detection of chIL-12 p70.

Chicken IL-23 p19 mRNA levels were measured in a broad range of chicken tissues and stimulated cells. The expression of chIL-23 p19 was ubiquitous and constitutive in the range of cells and tissues examined. Similar constitutive expression of huIL-23 p19 in a range of unstimulated tissues was seen in several genome-wide human tissue expression studies (Shmueli *et al.*, 2003; Yanai *et al.*, 2005), supporting the conclusion that chIL-23 may have a similar function to mammalian IL-23. Chicken

IL-12 p40 is ubiquitously expressed in a variety of tissues (Balu and Kaiser, 2003), whilst expression of chIL-12 p35 is more tightly controlled (Degen *et al.*, 2004), thus regulating the expression of functional IL-12. This is in contrast to mammals where IL-12 p35 is ubiquitously expressed and the expression of the p40 subunit is more tightly regulated, determining whether a cell produces functional IL-12 (Gately *et al.*, 1998). The co-expression of the IL-23 subunits, p19 and p40, would be expected in tissues as it would potentially allow the formation of IL-23. However, the ubiquitous expression of both subunits at the mRNA level suggests regulation of chIL-23 expression may be post-translational. The high mRNA levels seen in non-lymphoid tissues may in part be explained by the chicken's lack of lymph nodes and their development of diffuse lymphoid aggregates in organs (Michael and Hodges, 1974). Consequently, tissues defined in mammals as non-lymphoid contain more lymphoid cells in the chicken.

In contrast to being ubiquitously and highly expressed in a range of tissues, chIL-23 p19 mRNA expression was not up-regulated in stimulated splenocytes or bursal cells, and chIL-23 p19 expression in these cells declined over 24 h. Chicken IL-23 p19 was only up-regulated in stimulated thymocytes at 2 h post-stimulation. The predominant cell type in the thymus and bursa of Fabricius are T and B cells respectively, whereas the cell population in the spleen is more heterogeneous and includes APCs such as DCs and macrophages. IL-23 expression in mammals is mainly from DCs and tissue-resident macrophages through activation with TLR ligands such as LPS (Kastelein *et al.*, 2007). ConA, PHA and PMA and ionomycin were used to stimulate splenocytes, thymocytes and bursal cells respectively. These stimulants activate lymphocytes (Abb *et al.*, 1979; Dutton, 1972; Nowell, 1960),

resulting in expression of cytokines but there is no evidence to suggest they also stimulate DCs. ConA can activate macrophages to produce pro-inflammatory cytokines (Nugent *et al.*, 1985; Wang and Basch, 1979) but the expression of IL-23 in mammalian macrophages stimulated with ConA has not been investigated. Holt *et al.* (2011) showed *in vitro* stimulation of zebrafish leucocytes with LPS for 1 h increased expression of IL-23 p19. As the stimulants used here do not up-regulate expression of chIL-23 p19, one of the next steps would be to investigate the effects of stimulation with LPS or other mitogens on chicken splenocytes. In addition, stimulation of chicken bone marrow-derived macrophages with TLR ligands and subsequent measurement of chIL-23 p19 mRNA expression would be interesting. Finally, the development of a mAb which recognises chIL-23 p19 would also allow identification of cells expressing chIL-23 p19 and distinguish between the expression of chIL-12 p70 and chIL-23.

To summarise, IL-23 p19 has been identified and cloned in the chicken. Purified rchIL-23 was expressed from an IL-23 flexi-construct and the bioactivity of the recombinant protein demonstrated by increased mRNA levels of chIL-17A, chIL-17F and chIL-22 in splenocytes stimulated with rchIL-23. The rchIL-23 was recognised by chIL-12 mAbs against the chIL-12/chIL-23 p40 subunit. Chicken IL-23 p19 was expressed in a wide range of tissues but was not consistently up-regulated in stimulated splenocytes, thymocytes or bursal cells. The identification of IL-23 in the chicken and expression of purified rchIL-23 provides useful tools to further investigate the role of IL-23 and Th17 immune responses in the chicken.

Chapter 4 Th17 effector cytokine expression

4.1 Introduction

Th17 cells in mammals are characterised by the expression of the cytokines IL-17A, IL-17F, IL-21 and IL-22 (Korn *et al.*, 2009). The roles of the mammalian Th17 cytokines are discussed in Chapter 1. The Th17 effector cytokines IL-17A (Min and Lillehoj, 2002), IL-17F (Kim *et al.*, 2012b), IL-21 (Rothwell *et al.*, 2012) and IL-22 (Kim *et al.*, 2012a) have now been identified in the chicken. Relatively little is known about what cells express these cytokines and their anatomical location. Further, the expression and biological function of these Th17 effector cytokines during infection has not been investigated in the chicken. In mammals, the expression of Th17 cytokines is driven by cytokines, such as IL-23. The role of mammalian IL-23, and the influence it has on the immune response, particularly during persistent infections, has been well studied (Reviewed by Tato and Cua (2008)). However, expression of IL-23 in the chicken has not been studied to date.

Of the Th17 effector cytokines, the most studied outside of biomedical model species is IL-17A. However, despite being identified in several mammalian species (Katoh *et al.*, 2004; Riollet *et al.*, 2006; Tompkins *et al.*, 2010), birds (Min and Lillehoj, 2002; Yoo *et al.*, 2009) and fish (Gunimaladevi *et al.*, 2006; Kono *et al.*, 2011), information on the role of IL-17A in these species remains limited (reviewed by Mensikova *et al.*, 2013). Chicken IL-17A was identified by Min and Lillehoj (2002). Its role in the immune response has primarily been investigated in *Salmonella* and *Eimeria* infections. Chicken IL-17A mRNA levels are increased during *Salmonella enteritidis* infection (Matulova *et al.*, 2013). Further, mRNA expression of chIL-17A

is higher in non-vaccinated chickens compared to vaccinated birds following infection with *Salmonella enteritidis* (Matulova *et al.*, 2012). In *Eimeria tenella*-infected birds, chIL-17A is associated with increased immunopathology (Zhang *et al.*, 2013). Infected birds treated with chIL-17A showed exacerbated lesion scores and reduced body weight gain compared to untreated birds. The converse was seen in chIL-17A-neutralised birds (Zhang *et al.*, 2013). Infected birds treated with chIL-17A Ab showed reduced caecal lesion scores and increased body weight gain compared to untreated, infected birds. These studies suggest IL-17A in chickens plays a role in mucosal immunity and associated gut pathology as in mammals.

Chicken IL-17F was cloned from ConA-stimulated splenocytes (Kim *et al.*, 2012b). Increased chIL-17F mRNA expression was seen in *Eimeria*-infected birds compared to uninfected controls up to 10 dpi (Kim *et al.*, 2012b). Fish species possess genes with homology to mammalian IL-17A and IL-17F, which have been termed IL-17A/F (reviewed by Kono *et al.*, 2011). The functional role of IL-17F in other species remains to be investigated.

IL-21 has pleiotropic effects on both innate and adaptive immune responses. Beyond biomedical model species, it has been identified in cattle (Muneta *et al.*, 2003), pig (Muneta *et al.*, 2004) and fish (Wang *et al.*, 2011). Chicken IL-21 was cloned (Rothwell *et al.*, 2012) and, similarly to mammals, synergistically enhances T cell proliferation and inhibits maturation of DCs. Its expression and potential role in chicken immune responses, particularly Th17 immune responses, remains to be elucidated.

IL-22 has been identified in cattle and is expressed by $\alpha\beta$ and $\gamma\delta$ T cells (Ma *et al.*, 2010). Identification and demonstration of bioactivity of recombinant IL-22 in a non-mammalian species was initially made in fish (Igawa *et al.*, 2006; Monte *et al.*, 2011). IL-22 was identified in the chicken genome by Kaiser *et al.* (2005) and subsequently Kim *et al.* (2012a) demonstrated recombinant chIL-22 induced pro-inflammatory cytokines and anti-microbial peptides in epithelial cells and hepatocytes, similarly to mammalian IL-22.

The aims of this Chapter are to characterise Th17 immune responses in the chicken during infection by measuring mRNA levels of Th17 effector cytokines and IL-23 in various tissues, stimulated cells and during infection. The four disease models used in this Chapter to investigate Th17 immune responses in the chicken are discussed in Chapter 1.

4.2 Methods

General methods are outlined in Chapter 2. Additional methods and alterations are detailed here.

4.2.1 Cloning of *chIL-17F* cDNA

At the beginning of this study, the chIL-17F cDNA sequence was incorrectly annotated in the chicken genome (WASHUC2, Ensembl). The annotated sequence for chIL-17F in Ensembl (ENSGALT00000026912) appeared to be missing the first exon (Figure 4.1), suggesting the correct chIL-17F sequence to be the GenScan prediction (GENSCAN00000013648).

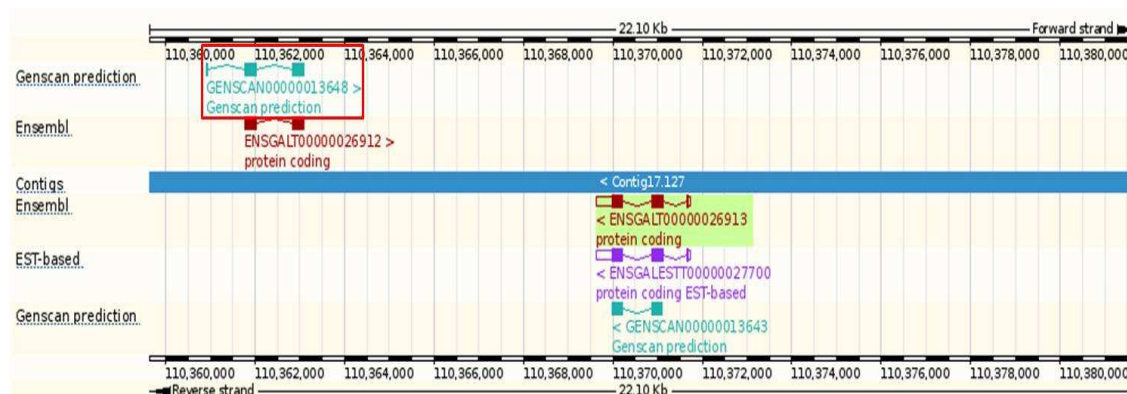


Figure 4.1. Ensembl (WASHUC2) showing the genetic location (red box) of chIL-17F (GENSCAN00000013648) on chromosome 3 of the chicken genome. The annotated gene for chicken IL-17F in Ensembl is located just below (ENSGALT00000026912).

Using the GenScan prediction for chIL-17F (GENSCAN00000013648) (Figure 4.1), forward and reverse primers (Table 2.1) were designed to amplify the full-length chIL-17F cDNA. RNA isolated from chicken splenocytes stimulated with ConA for 2 h was used as a template to amplify chIL-17F cDNA by PCR (section 2.4.3.1). The chIL-17F cDNA clone was TA-cloned into pGEM®-T Easy and sequenced to confirm the identity of chIL-17F and its similarity to mammalian IL-17F.

4.2.2 RT-qPCR (TaqMan®) primer and probe design

Having confirmed the full-length nt sequence of chIL-17F, primers and probe for use in RT-qPCR (TaqMan®) were designed using Primer Express v3 (Applied Biosystems) (Table 2.4). TaqMan® primers and probes were also designed for chIL-17A using Primer Express v3. Primers and probes to detect mRNA expression of chIL-21 (Rothwell *et al.*, 2012) and chIL-22 (Pathania, 2008) were designed previously. For all TaqMan® primer and probe sequences see Table 2.4.

4.3 Results

4.3.1 Identification and cloning of *chIL-17F*

Based on the predicted *chIL-17F* nt sequence (GENSCAN00000013648), primers IL-17F_F3 and IL-17F_R2 (Table 2.1) were designed to clone the predicted full-length *chIL-17F* cDNA, using cDNA prepared from ConA-stimulated splenocytes as a template. Figure 4.2 shows the product amplified with primers IL-17F_F3 and IL-17F_R2. It is approximately 500 nt in size and was then TA-cloned into pGEM®-T Easy and transformed into *E. coli* JM109 competent cells for subsequent plasmid isolation and sequencing.

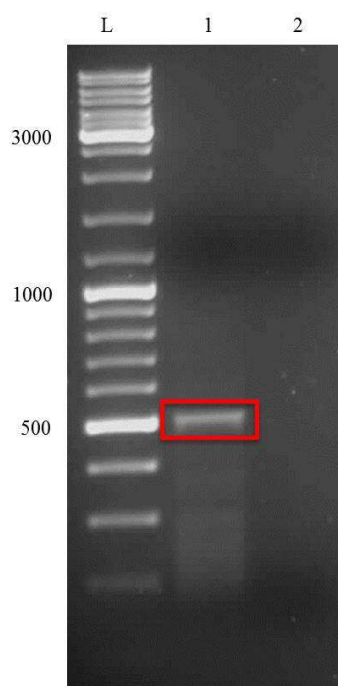


Figure 4.2. RT-qPCR amplification of the predicted *chIL-17F* cDNA. Lane L: GeneRuler™ DNA Ladder Mix (Thermo Scientific). Lane 1: cDNA from ConA-stimulated (2 h) splenocytes. Lane 2: negative control. Red box highlights PCR product selected for TA cloning and sequencing.

Multiple cloned plasmids were sequenced to confirm the nt sequence of *chIL-17F*, which also confirmed the misannotation of the *chIL-17F* gene

(ENSGALT00000026912) in the Ensembl database (WASHUC2). Alignment of the misannotated chIL-17F aa sequence and the cloned and translated chIL-17F aa sequence (Figure 4.3), confirmed that the additional 5' exon of the chIL-17F GenScan prediction (GENSCAN00000013648) encodes a start codon and 8 additional aa.

```

predicted chIL-17F : -----FRSLLLVLTTLTVWSSPHGKVVPRPRKDGSEKLSIEDCL : 41
actual chIL-17F   : MAFASCAAVFRSLLLVLTTLTVWSSPHGKVVPRPRKDGSEKLSIEDCL : 50

predicted chIL-17F : NQKDPSFPTMVKVDIRIGSSDPASRMIDIRNRSLAPWNYRLDEDPNRF : 91
actual chIL-17F   : NQKDPSFPTMVKVDIRIGSSDPASRMIDIRNRSLAPWNYRLDEDPNRF : 100

predicted chIL-17F : QVIADAECRLLGCLNSLGQEDRSLNSVPITQEIILVLRREQRCQPTYHLE : 141
actual chIL-17F   : QVIADAECRLLGCLNSLGQEDRSLNSVPITQEIILVLRREQRCQPTYHLE : 150

predicted chIL-17F : KKLI TVGCTCAAPVIQHQS : 160
actual chIL-17F   : KKLI TVGCTCAAPVIQHQS : 169

```

Figure 4.3. Comparison of the annotated chIL-17F aa sequence (ENSGALT00000026912) and the GenScan prediction for chIL-17F (GENSCAN00000013648). The GenScan prediction encodes an additional start codon and 8 aa.

4.3.2 Structural features of chIL-17F

The chIL-17F cDNA encodes a predicted protein of 169 aa (Figure 4.4), which contains a predicted 25 aa signal peptide with a resultant mature protein of 144 aa. This has 39.6% identity at the predicted aa level (67/169 differences) compared with human, mouse and cattle IL-17F. Further, there are 49.1%, 50.3% and 50.8% similarity between chIL-17F and human, mouse and cattle IL-17F respectively. There are seven cysteine residues in chIL-17F, of which six are conserved with mammals, in which there are two disulphide bonds (indicated by solid light grey lines). Positively charged arginine residues involved in the interaction with the IL-17-RA are conserved in the chicken. The location of an α -helix and five β -strands in human IL-17F are shown as an open arrow and dark grey arrows respectively above the aa sequences. Mammalian IL-17s display a cysteine knot structure. There are two

conserved serine residues which replace the cysteine residues in the position of the third disulphide bond, within the cysteine knot. One of these serine residues (Ser¹²⁶) is conserved in the chicken but the other is not (Ala⁸⁶).

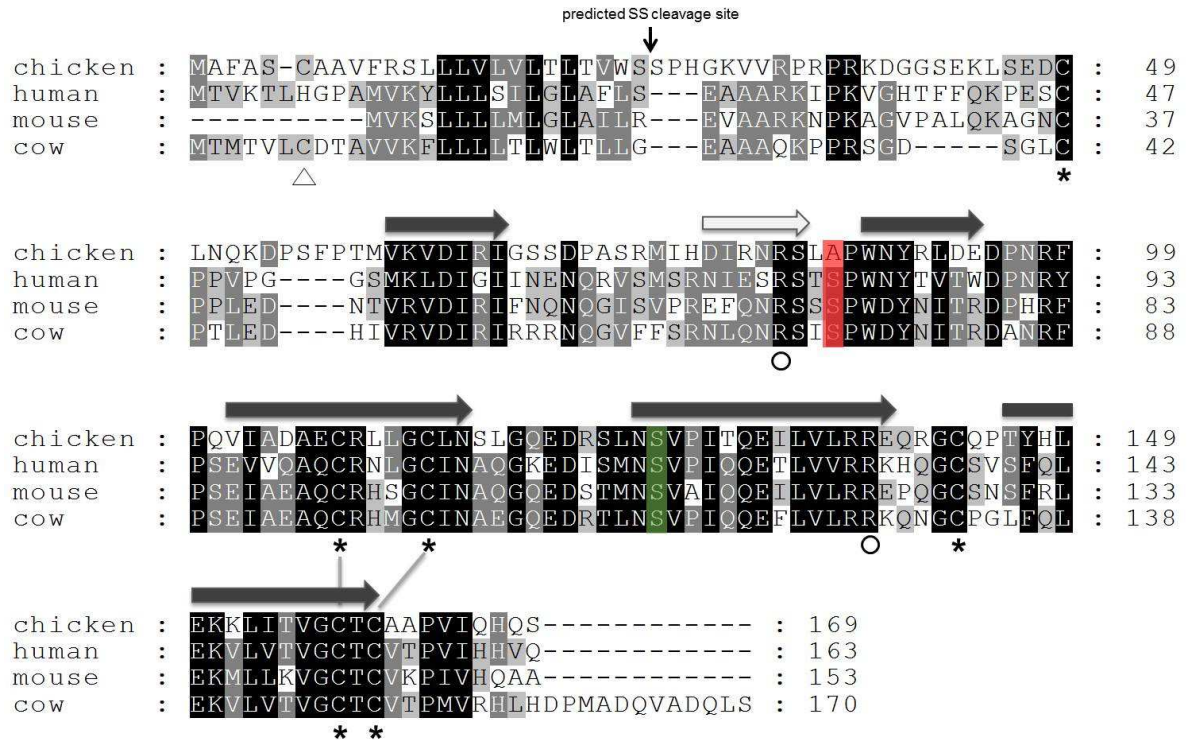


Figure 4.4. Comparison of the chIL-17F aa sequence with human, mouse and cattle IL-17F. Shaded areas represent conservation of aa similarity, the darker the shading, the more conserved the residue is across the species. The vertical black arrow indicates the predicted signal sequence cleavage site of chIL-17F. Cysteine residues conserved across all species are indicated by asterisks. The chicken-specific cysteine residue is identified by a triangle. Conserved positively charged arginine residues involved in the interaction with IL-17-RA are indicated by small circles. An α -helix is shown by an open arrow, β -strands by dark grey arrows. Disulphide bonds are indicated by solid grey lines connecting the relevant cysteines. In mammalian IL-17s there are two conserved serines which replace the cysteines in the position of the third disulphide bond in the cysteine knot. The serine residue conserved in the chicken is highlighted in green and that conserved only in mammals is highlighted in red.

4.3.3 Analysis of chicken Th17 effector cytokine mRNA expression in tissues and stimulated cells

The mRNA expression of chicken IL-17A, IL-17F, IL-21 and IL-22 was studied in a wide range of chicken tissues and stimulated cells in order to compare it to cytokine expression in mammals and fish.

4.3.3.1 *chIL-17A*

As shown in Figure 4.5, *chIL-17A* mRNA is widely expressed in all immune and non-immune tissues investigated at moderate levels. The highest mRNA levels in non-lymphoid tissues were seen in the kidney and in lymphoid tissues in the spleen, with very similar levels seen in the thymus and Meckel's diverticulum.

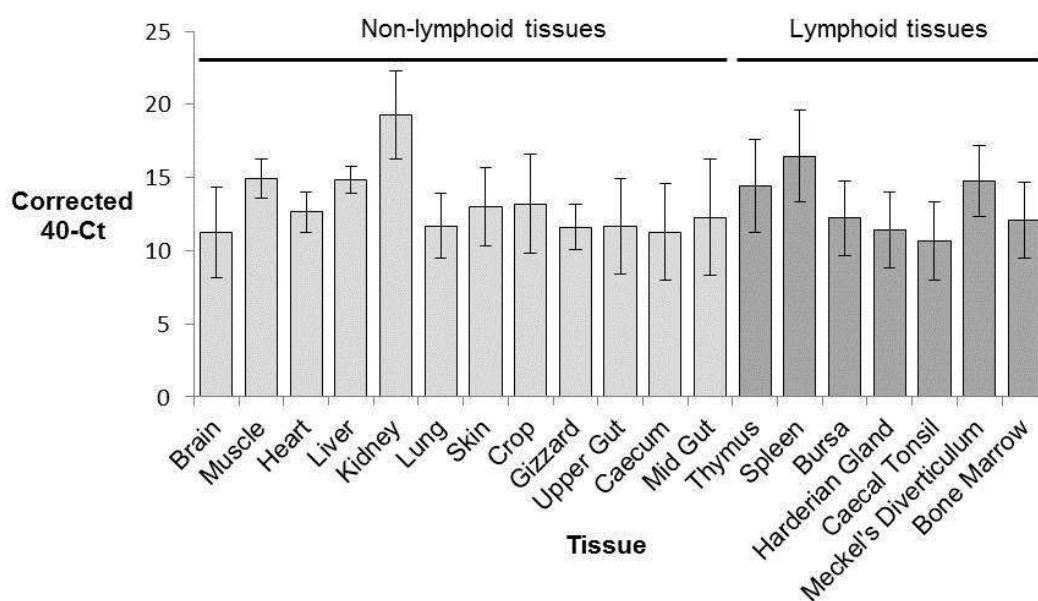


Figure 4.5. Expression patterns of *chIL-17A* mRNA in tissues as measured by RT-qPCR. Data are expressed as corrected 40-Ct values \pm SEM. Assays were carried out in triplicate and each value is the mean of 3 birds.

Levels of chIL-17A mRNA were also measured in stimulated lymphoid cells and compared to those in unstimulated controls. Splenocytes were stimulated with ConA, thymocytes with PHA and bursal cells with PMA and ionomycin over a time-course of 2-24 h. As seen in Figure 4.6 (A), ConA stimulation significantly up-regulated ($p = 0.002$) chIL-17A mRNA expression in splenocytes after 6h of stimulation. There is a significant negative linear relationship ($p = 0.018$) in fold change over time of chIL-17A mRNA levels in stimulated splenocytes. Chicken IL-17A mRNA levels decreased from 2-24 h. There was no difference in mRNA expression between control and stimulated cells in the thymus at any time-point observed (Figure 4.6 (B)). Chicken IL-17A mRNA levels in stimulated bursal cells were significantly up-regulated at 2 h post-stimulation (Figure 4.6 (C)), and showed a significant negative linear decline ($p < 0.001$) in fold change over time, decreasing from 2-24 h post-stimulation.

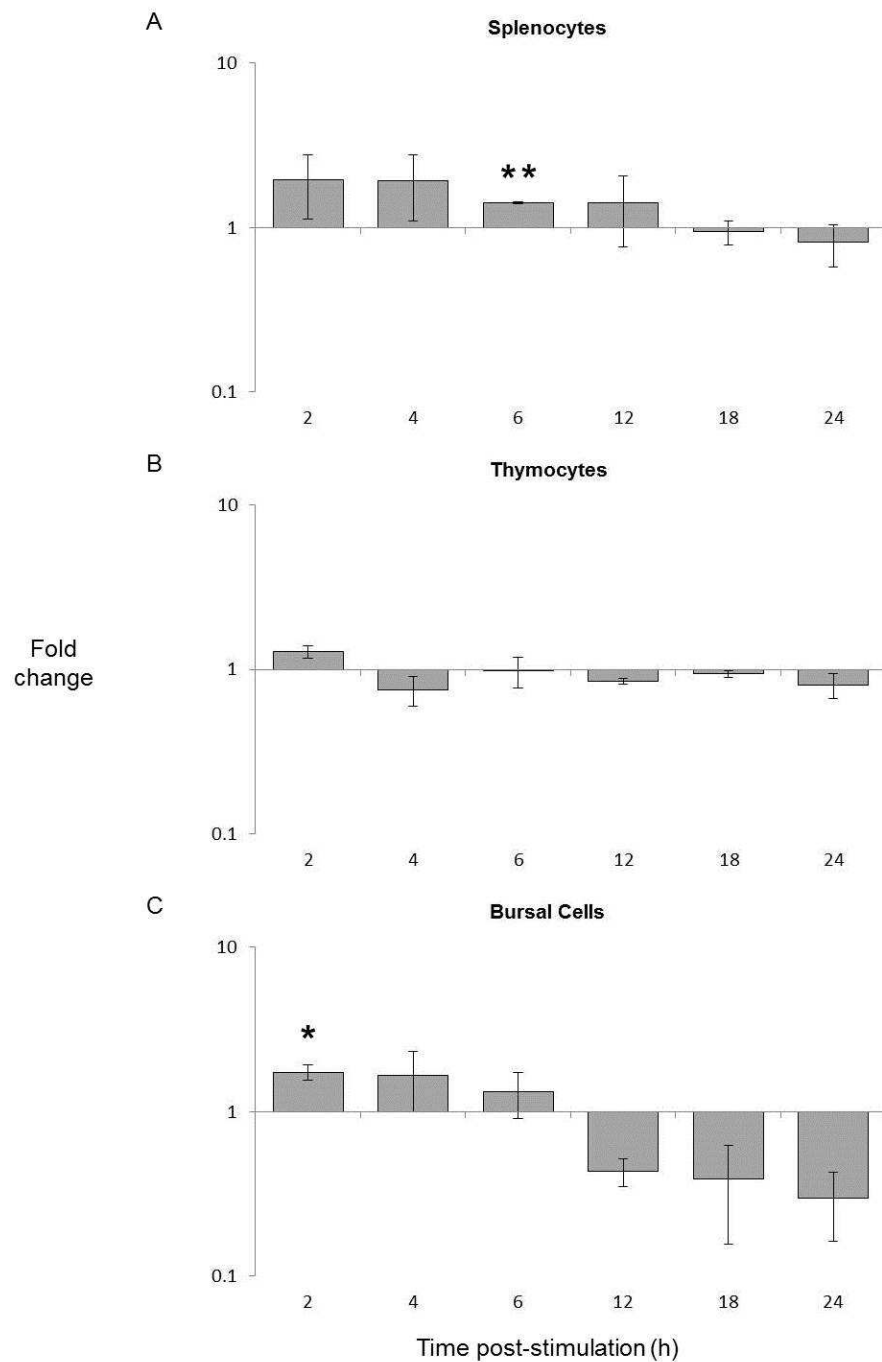


Figure 4.6. Expression patterns of chIL-17A mRNA in stimulated (A) splenocytes, (B) thymocytes and (C) bursal cells. Data are expressed as fold change compared to levels in age-matched unstimulated controls \pm SEM. A linear mixed effect model was used to determine if there was a relationship between mRNA levels and time. A standard one-sample *t* test was performed to determine if cytokine levels were significantly up or down-regulated relative to a fold change of one as indicated by a single asterisk ($p < 0.05$) or double asterisk ($p < 0.01$). Chicken splenocytes, thymocytes and bursal cells were stimulated with ConA, PHA and PMA and ionomycin respectively for between 2-24 h. Assays were carried out in triplicate and each value is the mean of 3 birds.

4.3.3.2 Chicken IL-17F

As shown in Figure 4.7, chIL-17F mRNA is widely expressed in tissues albeit at a low level and also at slightly lower levels compared to chIL-17A (Figure 4.5). The highest levels of chIL-17F mRNA expression in non-lymphoid tissues were in the lung and in lymphoid tissues were in the caecal tonsil and the bursa of Fabricius.

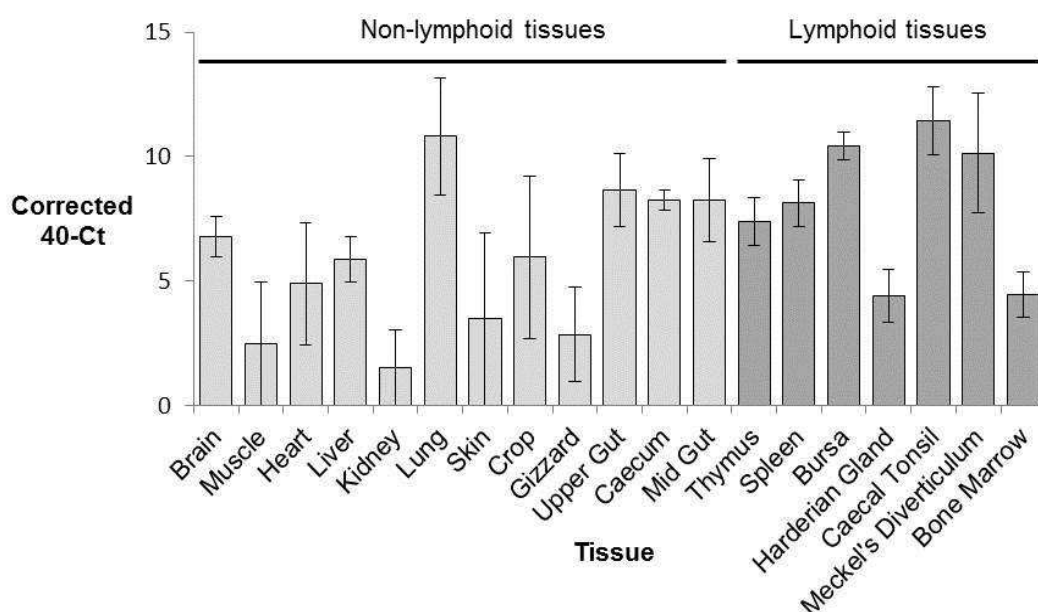


Figure 4.7. Expression patterns of chIL-17F mRNA in tissues as measured by RT-qPCR. Data are expressed as corrected 40-Ct values \pm SEM. Assays were carried out in triplicate and each value is the mean of 3 birds.

Chicken IL-17F mRNA levels were also measured in stimulated lymphoid cells and compared to those in unstimulated controls. Chicken IL-17F mRNA was significantly up-regulated at 6-18 h ($p < 0.034$) post-stimulation in ConA-stimulated splenocytes (Figure 4.8 (A)). However, there was no significant relationship in chIL-17F mRNA expression in stimulated splenocytes over time. Chicken IL-17F mRNA in control and stimulated thymocytes was extremely low and there was no difference in mRNA levels between control and stimulated cells in the thymus at any time-point (Figure 4.8 (B)). There was no significant relationship in chIL-17F mRNA expression in stimulated thymocytes over time. Expression of chIL-17F mRNA in stimulated bursal cells was significantly up-regulated between 2-6 h ($p < 0.026$) post-stimulation (Figure 4.8 (C)). Chicken IL-17F mRNA levels also showed a significant negative quadratic relationship ($p = 0.041$) in fold change over time. Levels of chIL-17F mRNA compared to unstimulated controls initially decreased over time (2-18 h) before increasing at 24 h.

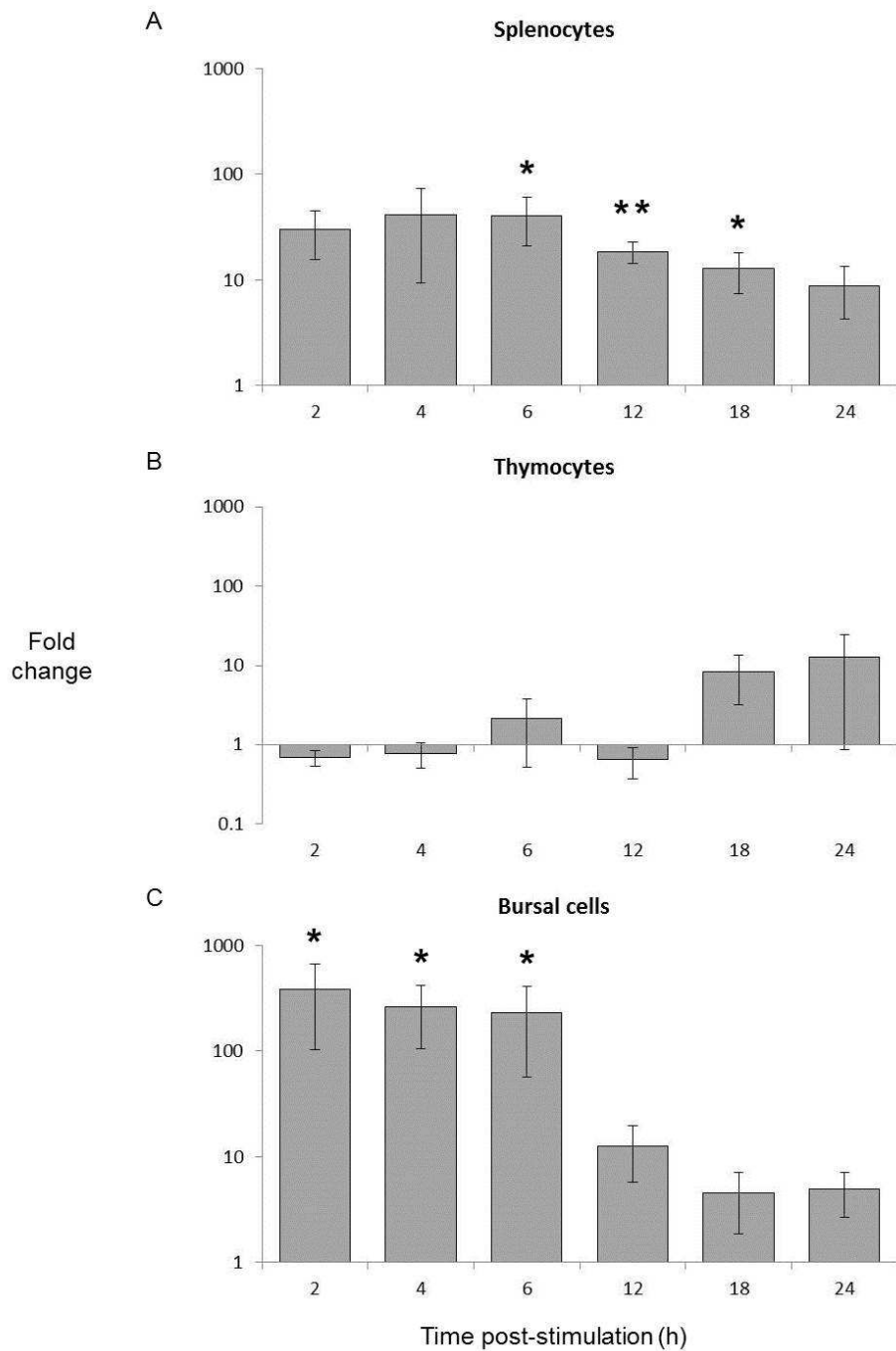


Figure 4.8. Expression patterns of chIL-17F mRNA in stimulated (A) splenocytes, (B) thymocytes and (C) bursal cells. Data are expressed as fold change compared to levels in age-matched unstimulated controls \pm SEM. A linear mixed effect model was used to determine if there was a relationship between mRNA levels and time. A standard one-sample *t* test was performed to determine if cytokine levels were significantly up or down-regulated relative to a fold change of one as indicated by a single asterisk ($p < 0.05$) or double asterisk ($p < 0.01$). Chicken splenocytes, thymocytes and bursal cells were stimulated with ConA, PHA and PMA and ionomycin respectively for between 2-24 h. Assays were carried out in triplicate and each value is the mean of 3 birds.

4.3.3.3 Chicken IL-21

Figure 4.9 shows low levels of chIL-21 mRNA detected in a variety of tissues. The highest chIL-21 mRNA levels in non-lymphoid tissues were measured in the gut and no expression was detected in muscle or skin. In lymphoid tissues, the highest chIL-21 mRNA levels were in Meckel's diverticulum, bursa of Fabricius and caecal tonsil.

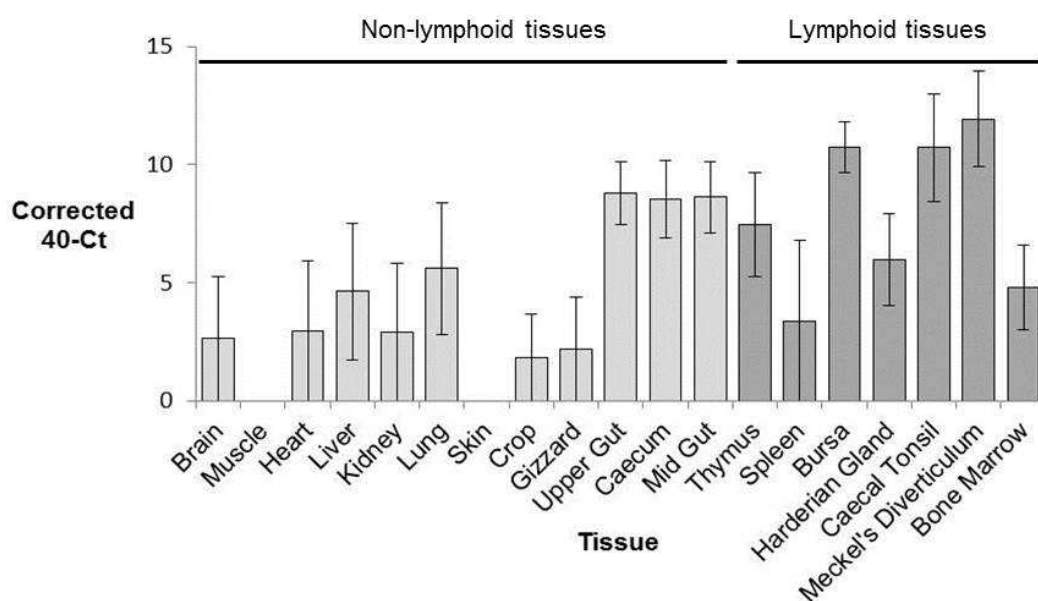


Figure 4.9. Expression patterns of chIL-21 mRNA in tissues as measured by RT-qPCR. Data are expressed as corrected 40-Ct values \pm SEM. Assays were carried out in triplicate and each value is the mean of 3 birds.

Messenger RNA levels of chIL-21 were also measured in unstimulated and stimulated lymphoid cells by RT-qPCR. Chicken IL-21 mRNA in stimulated splenocytes was significantly up-regulated compared to unstimulated controls at 2-6 h ($p < 0.048$) post-stimulation (Figure 4.10 (A)). Chicken IL-21 mRNA expression in stimulated splenocytes also showed a significant positive linear relationship ($p = 0.023$) over time, increasing from 2-24 h post-stimulation. Chicken IL-21 mRNA expression in stimulated thymocytes was only measured at 2 h post-stimulation; the increase in chIL-21 mRNA levels was not significant (Figure 4.10 (B)). Stimulated bursal cells showed a significant increase in chIL-21 mRNA compared to unstimulated controls at 2, 6 and 12 h ($p < 0.008$) post-stimulation (Figure 4.10 (C)). Further, there was a significant negative quadratic relationship in fold change of chIL-21 mRNA expression compared to unstimulated controls over time ($p = 0.049$). Chicken IL-21 mRNA levels in stimulated bursal cells increased from 2-6 h post stimulation and then decreased from 6-24 h post stimulation.

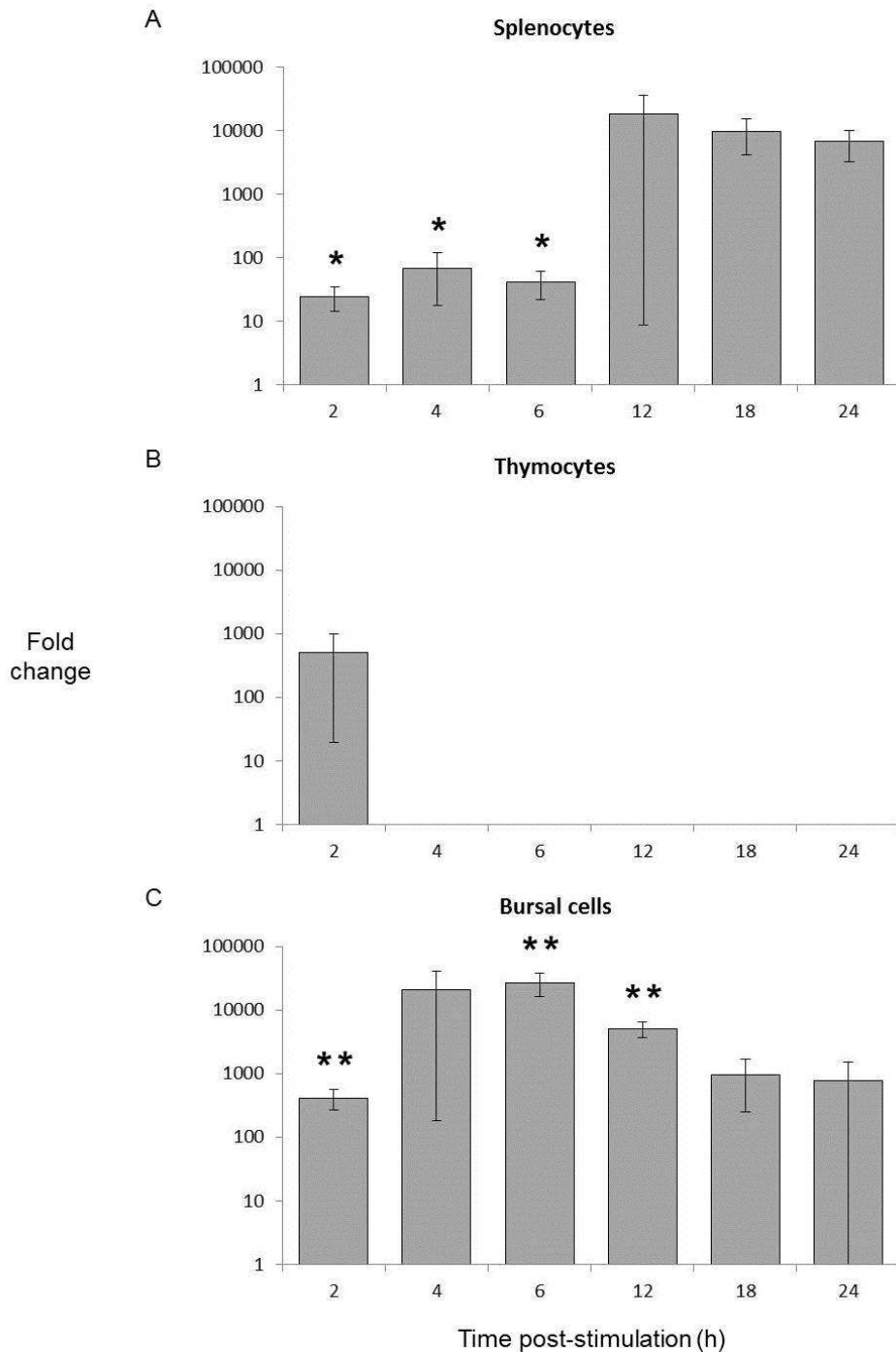


Figure 4.10. Expression patterns of chIL-21 mRNA in stimulated (A) splenocytes, (B) thymocytes and (C) bursal cells. Data are expressed as fold change compared to levels in age-matched unstimulated controls \pm SEM. A linear mixed effect model was used to determine if there was a relationship between mRNA levels and time. A standard one-sample *t* test was performed to determine if cytokine levels were significantly up or down-regulated relative to a fold change of one as indicated by a single asterisk ($p < 0.05$) or double asterisk ($p < 0.01$). Chicken splenocytes, thymocytes and bursal cells were stimulated with ConA, PHA and PMA and ionomycin respectively for between 2-24 h. Assays were carried out in triplicate and each value is the mean of 3 birds.

4.3.3.4 *Chicken IL-22*

RT-qPCR was used to measure mRNA levels of chIL-22 in various tissues and stimulated lymphoid cells. As shown in Figure 4.11, chIL-22 mRNA is widely expressed in tissues but at very low levels. The highest mRNA levels in non-lymphoid cells were measured in muscle, heart and kidney. The highest mRNA expression of chIL-22 in lymphoid cells was in caecal tonsil.

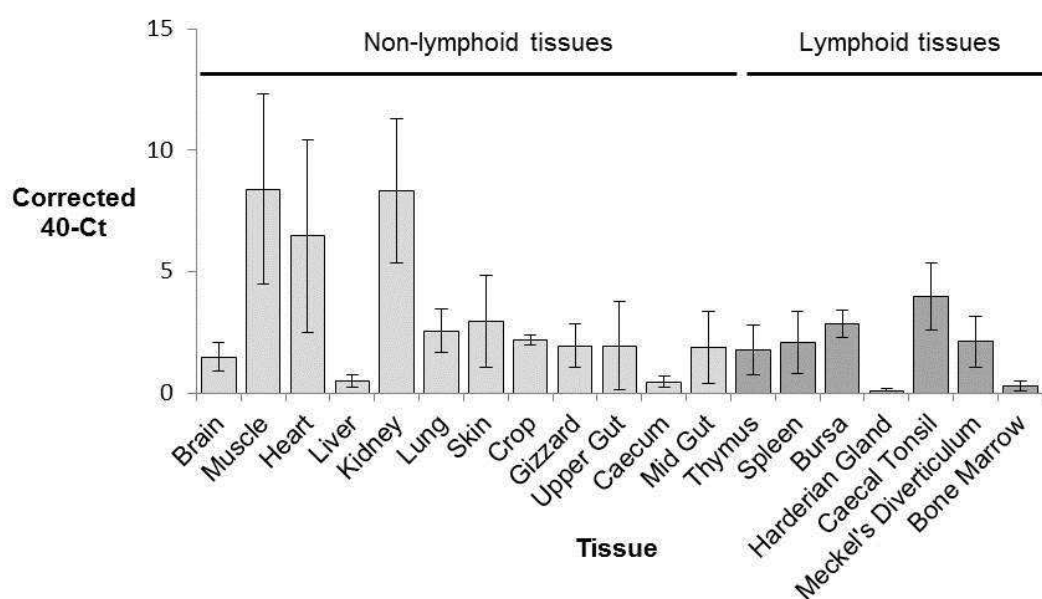


Figure 4.11. Expression patterns of chIL-22 mRNA in tissues as measured by RT-qPCR. Data are expressed as corrected 40-Ct values \pm SEM. Assays were carried out in triplicate and each value is the mean of 3 birds.

Chicken IL-22 mRNA was measured in stimulated lymphoid tissues and compared to unstimulated controls. Chicken IL-22 mRNA levels were measured in stimulated splenocytes at all time-points but there was no significant up-regulation in stimulated cells compared to levels in unstimulated controls, nor was there a significant relationship in fold change of chIL-22 mRNA expression compared to unstimulated controls over time (Figure 4.12 (A)). No chIL-22 mRNA was detected in control or stimulated thymocytes (Figure 4.12 (B)). Stimulated bursal cells expressed increased levels of chIL-22 mRNA compared to those in unstimulated controls at 2, 4, 6 and 12 h stimulation but the levels were not significant (Figure 4.12 (C)). Chicken IL-22 mRNA levels were not measured at 18 or 24 h post-stimulation. There was not a significant relationship in fold change of chIL-22 mRNA levels compared to unstimulated controls over time in stimulated bursal cells.

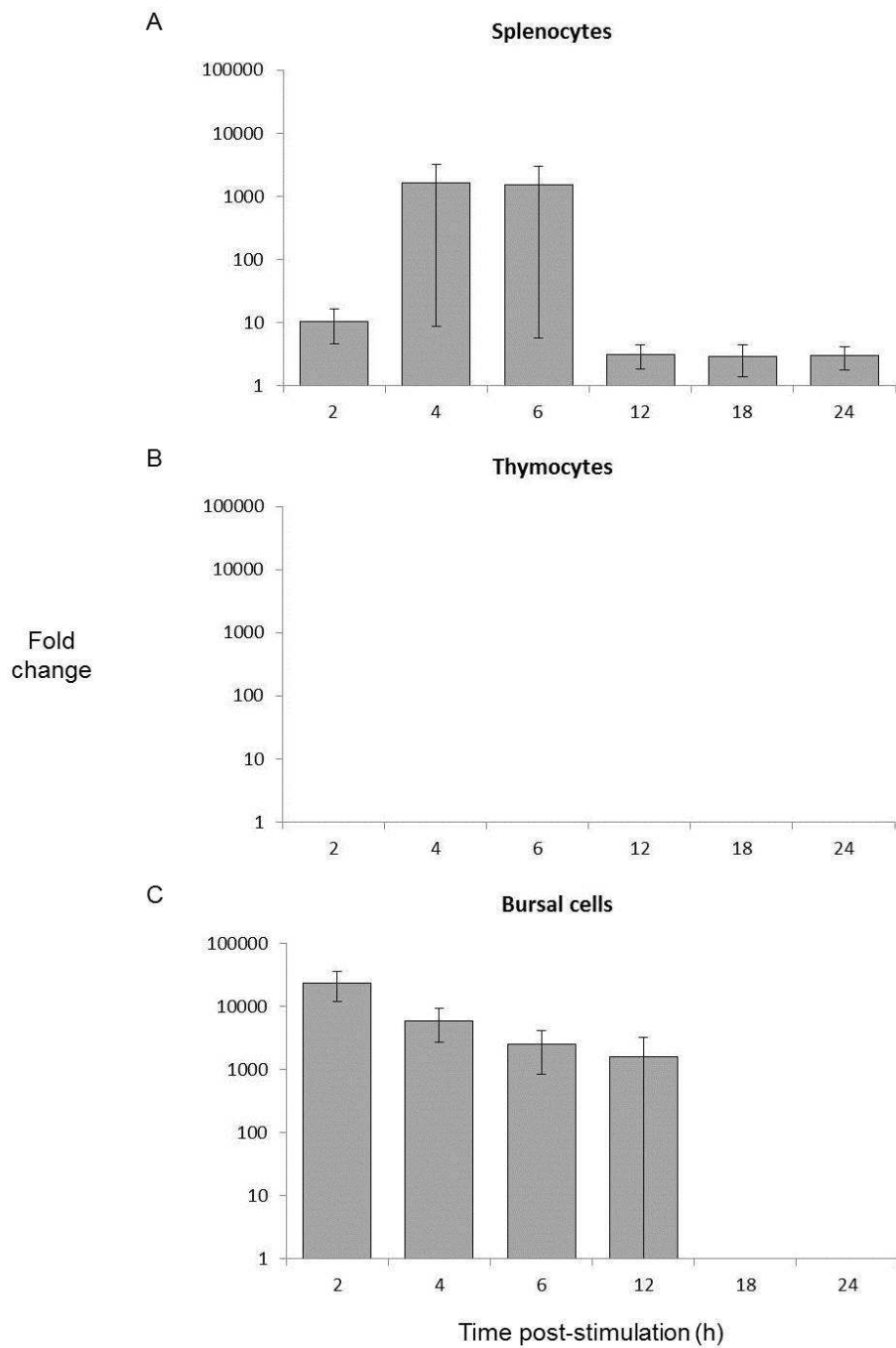


Figure 4.12. Expression patterns of chIL-22 mRNA in stimulated (A) splenocytes, (B) thymocytes and (C) bursal cells. Data are expressed as fold change compared to levels in age-matched unstimulated controls \pm SEM. A linear mixed effect model was used to determine if there was a relationship between mRNA levels and time. A standard one-sample *t* test was performed to determine if cytokine levels were significantly up or down-regulated relative to a fold change of one. Chicken splenocytes, thymocytes and bursal cells were stimulated with ConA, PHA and PMA and ionomycin respectively for between 2-24 h. Assays were carried out in triplicate and each value is the mean of 3 birds.

4.3.4 Analysis of chicken Th17 cytokine mRNA expression in vivo following bacterial, viral and parasitic challenge

To further investigate whether chickens mount Th17 immune responses, mRNA levels of the Th17 effector cytokines: chIL-17A, chIL-17F, chIL-21 and chIL-22 were measured during Marek's disease, Infectious bursal disease and histomoniasis infections. The cytokines chIL-17A, chIL-17F and chIL-23 p19 were measured during *S. aureus* infection (section 2.2).

4.3.4.1 Marek's disease

The Th17 effector cytokines, chIL-17A, chIL-17F, chIL-21 and chIL-22 as well as chIL-23 p19 mRNA expression was measured in the spleen from a resistant and susceptible MDV-infected chicken line and compared to uninfected, age-matched controls.

Figure 4.13 (A) shows there was a significant interaction ($p = 0.001$) between the line of chicken and the infected/uninfected group in chIL-23 p19 mRNA expression. Infected resistant line 6₁ birds expressed lower levels of chIL-23 p19 mRNA compared to uninfected line 6₁ resistant birds, whereas infected susceptible line 7₂ birds expressed higher levels of chIL-23 p19 mRNA compared to uninfected line 7₂ birds at all time points. This significant interaction between the line of chicken and the infected/uninfected group and subsequent relationship was also measured in chIL-17A ($p < 0.001$) and chIL-21 ($p = 0.003$) mRNA levels (Figure 4.13 (B) & (C)).

Further, there was also a significant main effect of dpi ($p = 0.01$) and line ($p = 0.001$) on expression of chIL-21 mRNA (Figure 4.13 (C)). At 4 dpi, chIL-21 mRNA levels were higher compared to 2 and 3 dpi, irrespective of group (infected/uninfected) and

line of bird. Resistant line 6₁ birds expressed higher levels of chIL-21 mRNA compared to susceptible line 7₂ birds, irrespective of group (uninfected/infected) and dpi.

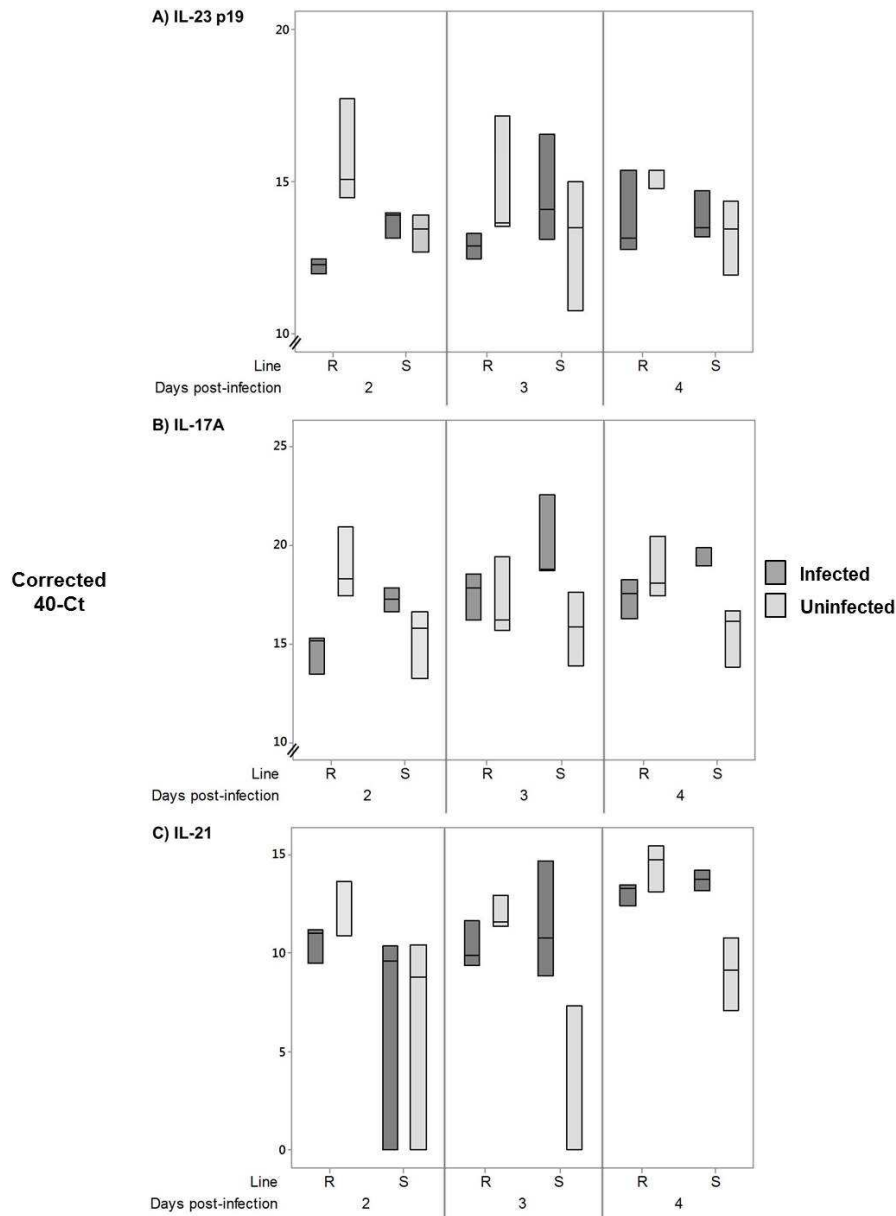


Figure 4.13. Quantification of A) chIL-23 p19, B) chIL-17A and C) chIL-21 mRNA levels in spleens during MDV infection. Data are expressed as corrected 40-Ct of infected and uninfected resistant (R) line 6₁ and susceptible (S) line 7₂ birds at 2, 3 and 4 days post-infection. Dark grey boxes represent infected birds (I) and light grey boxes represent uninfected birds (U). Significant interactions ($p < 0.05$) between groups of data were determined using analysis of variance. Assays were carried out in triplicate.

There was also a significant interaction ($p = 0.023$) between the dpi and the infected/uninfected group in chIL-17A mRNA expression (Figure 4.14). At 2 dpi, chIL-17A mRNA in MDV-infected birds was lower compared to mRNA levels in MDV-uninfected birds, whereas at 3 and 4 dpi, chIL-17A mRNA expression in MDV-infected birds was higher compared to those in age-matched, uninfected controls.

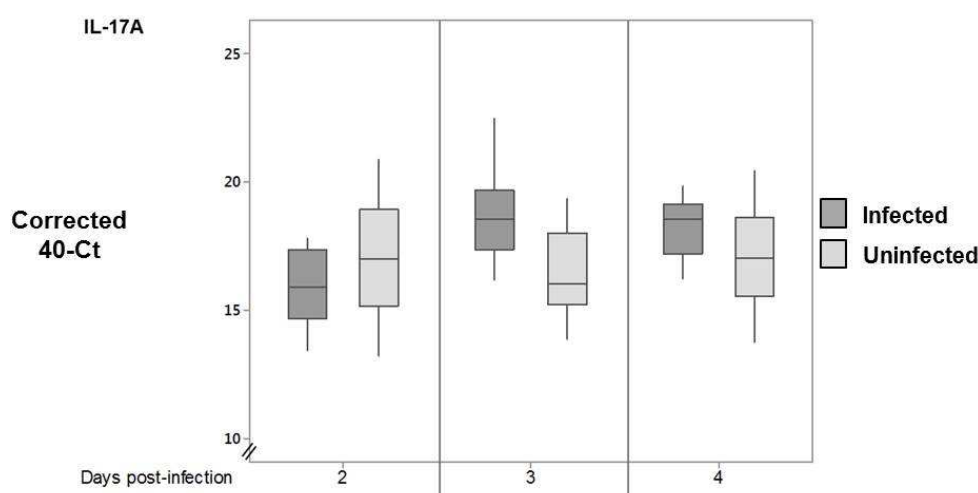


Figure 4.14. Quantification of chIL-17A mRNA levels in spleens during MDV infection. Data are expressed as corrected 40-Ct of infected and uninfected birds at 2, 3 and 4 days post-infection. Dark grey boxes represent infected birds (I) and light grey boxes represent uninfected birds (U). Significant interactions ($p < 0.05$) between groups of data were determined using analysis of variance. Assays were carried out in triplicate.

Chicken IL-17F mRNA levels showed a significant main effect ($p < 0.001$) between uninfected and infected birds, irrespective of the line of bird or the dpi (Figure 4.15). Expression of chIL-17F mRNA was higher in uninfected birds compared to MDV-infected birds.

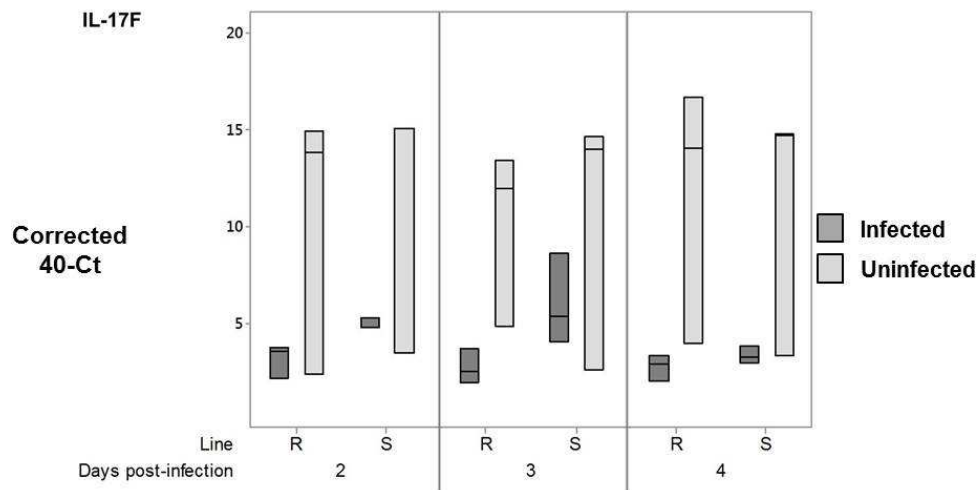


Figure 4.15. Quantification of chIL-17F mRNA levels in spleens during MDV infection. Data are expressed as corrected 40-Ct of infected and uninfected resistant (R) line 6₁ and susceptible (S) line 7₂ birds at 2, 3 and 4 days post-infection. Dark grey boxes represent infected birds (I) and light grey boxes represent uninfected birds (U). Significant interactions ($p < 0.05$) between groups of data were determined using analysis of variance. Assays were carried out in triplicate.

Expression of chIL-22 mRNA did not show any significant interactions between the line of bird, the sample type (infected/uninfected) or the dpi (Figure 4.16).

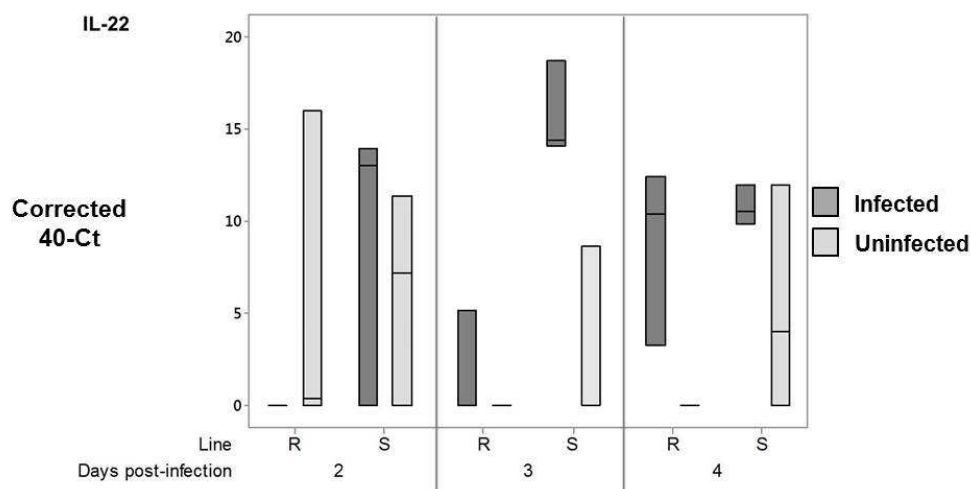


Figure 4.16. Quantification of chIL-22 mRNA levels in spleens during MDV infection. Data are expressed as corrected 40-Ct of infected and uninfected resistant (R) line 6₁ and susceptible (S) line 7₂ birds at 2, 3 and 4 days post-infection. Dark grey boxes represent infected birds (I) and light grey boxes represent uninfected birds (U). Significant interactions ($p < 0.05$) between groups of data were determined using analysis of variance. Assays were carried out in triplicate.

4.3.4.2 Infectious bursal disease

Using previously archived RNA samples, chIL-17A, chIL-17F, chIL-21 and chIL-22 mRNA levels were measured in caecal tonsils from IBDV-infected chickens and compared to uninfected, age-matched controls at 1, 3 and 4 dpi.

Chicken IL-17A mRNA levels were significantly up-regulated at 1 dpi ($p = 0.036$) but then down-regulated at 3 dpi ($p = 0.024$) (Figure 4.17 (A)). There was a significant decrease in chIL-17A mRNA expression at 3 and 4 dpi ($p < 0.03$) compared to 1 dpi. Messenger RNA levels of chIL-17F were significantly increased at 3 dpi compared to 1 dpi ($p = 0.019$) (Figure 4.17 (B)). Chicken IL-21 mRNA levels were significantly down-regulated at 4 dpi ($p = 0.033$) and significantly decreased at 4 dpi compared to 1 dpi ($p = 0.035$). Chicken IL-22 mRNA expression was significantly up-regulated at 4 dpi ($p = 0.026$) and chIL-22 mRNA expression at 1 and 3 dpi was significantly reduced compared to 4 dpi ($p < 0.03$) (Figure 4.17 (C) and (D)).

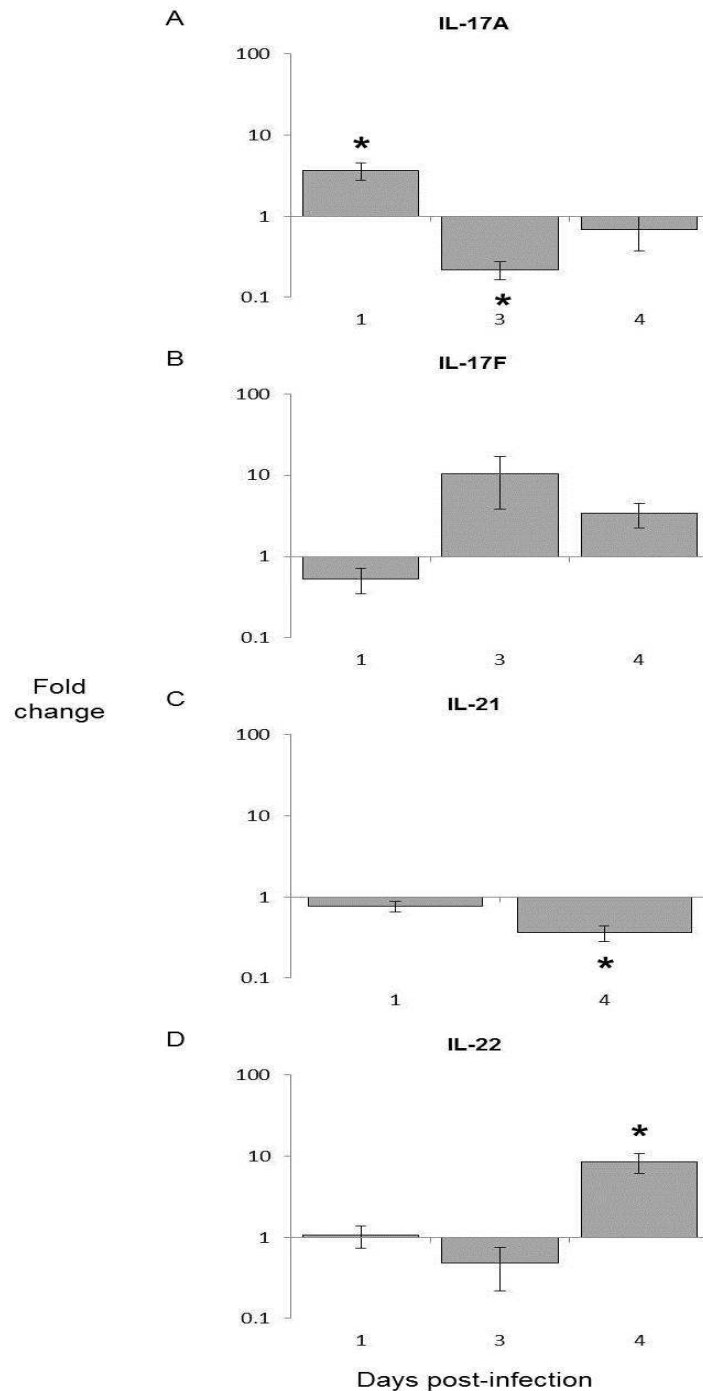


Figure 4.17. Quantification of A) chIL-17A, B) chIL-17F, C) chIL-21 & D) chIL-22 mRNA levels in caecal tonsils during IBDV infection. Data are expressed as fold change compared to levels in age-matched uninfected controls \pm SEM at 1, 3 and 4 days post-infection (dpi). A 1-way ANOVA and post-hoc standard Tukey test were used to determine if the mRNA levels at each dpi were significantly different to each other. A standard one-sample *t* test was performed to determine if cytokine levels were significantly up or down-regulated relative to a fold change of one ($p < 0.05$) as indicated by a single asterisk ($p < 0.05$). Each infected value is the mean of 3 birds. Control values are from 1 bird. Assays were carried out in triplicate.

4.3.4.3 *Histomoniasis*

Chicken IL-17A, chIL-17F, chIL-21 and chIL-22 mRNA expression was measured in caecal tonsils from *H. meleagridis*-infected chickens and compared to uninfected, age-matched controls over a time course from 1-30 dpi.

Chicken IL-17A and chIL-21 mRNA levels (Figure 4.18 (A) & (C)) were high at 1 dpi, decreasing until 12 dpi and then increasing from 18-30 dpi, demonstrating a quadratic relationship over time. This relationship is stronger for chIL-17A mRNA expression with an R^2 value of 34.4% for chIL-17A compared to 24% for chIL-21 mRNA expression. Chicken IL-17F and chIL-22 mRNA levels (Figure 4.18 (B) & (D)) were highest at 1 dpi, decreasing over time until 30 dpi, demonstrating a linear relationship over time. All relationships were significant ($p < 0.05$).

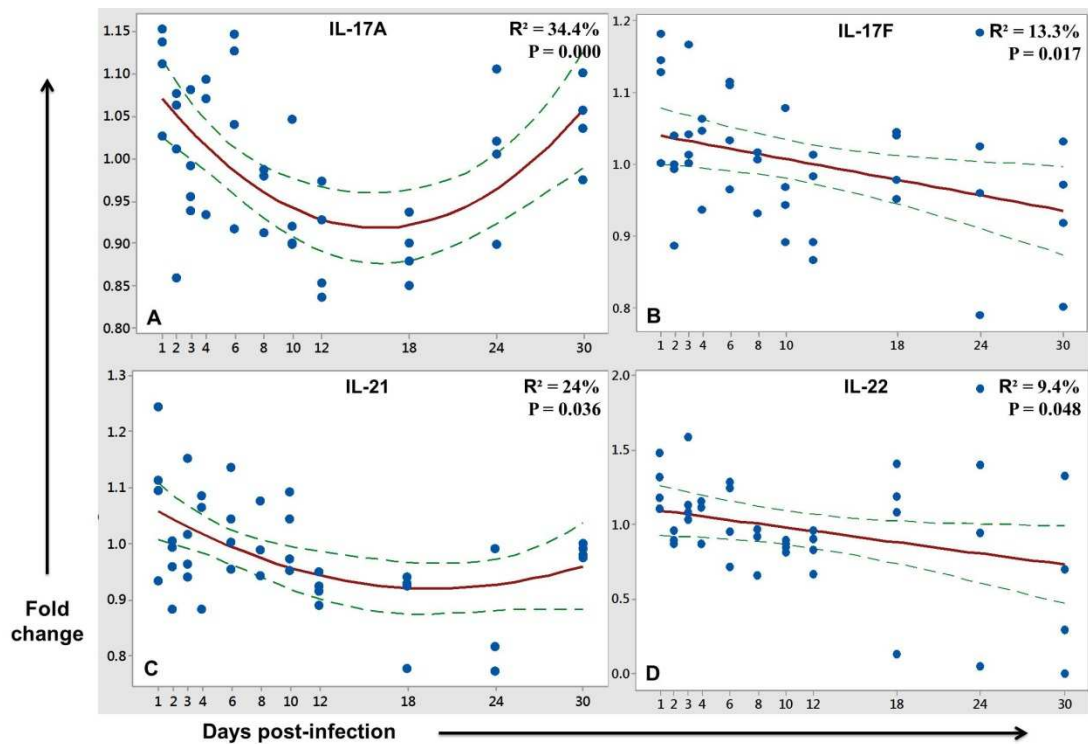


Figure 4.18. mRNA levels of (A) chIL-17A, (B) chIL-17F, (C) chIL-21 & (D) chIL-22 in caecal tonsils during *H. meleagridis* infection. Data are expressed as fold change compared to the mean value of 2 age-matched uninfected controls at 1, 2, 3, 4, 6, 8, 10, 12, 18, 24 and 30 days post-infection. Regression analysis was used to statistically analyse the data. Chicken IL-17A and chIL-21 mRNA expression show a quadratic relationship over time. Chicken IL-17F and chIL-22 mRNA expression show a linear relationship over time. The red line represents the regression analysis and the green dashed lines indicate the 95% confidence intervals. R² values and p values for each cytokine are indicated in the top right hand corner of each figure. All relationships have a significance value of less than 0.05 ($p < 0.05$).

4.3.4.4 *Staphylococcus aureus* pilot study

A *S. aureus* *in vivo* challenge experiment pilot study was carried out in collaboration with Ross Fitzgerald and Andreas Lengeling. RT-qPCR (TaqMan®) was used to measure mRNA expression of chIL-17A, chIL-17F and chIL-23 p19 from the spleens and bone marrow of *S. aureus*-infected chickens.

The mRNA expression of chIL-17A, chIL-17F and chIL-23 p19 was measured in the spleen and bone marrow at 6 and 11 dpi. There was no difference in fold change of chIL-17A mRNA in the spleen or chIL-17F mRNA in the spleen and bone marrow between the wild-type and mutant groups compared to unstimulated controls at any

time-point (Figure 4.19 (A) and (B)). Chicken IL-23 p19 mRNA levels were significantly decreased in the wild-type and mutant infected groups at 11 dpi compared to 6 dpi in the spleen and the bone marrow ($p < 0.026$) (Figure 4.19 (C)).

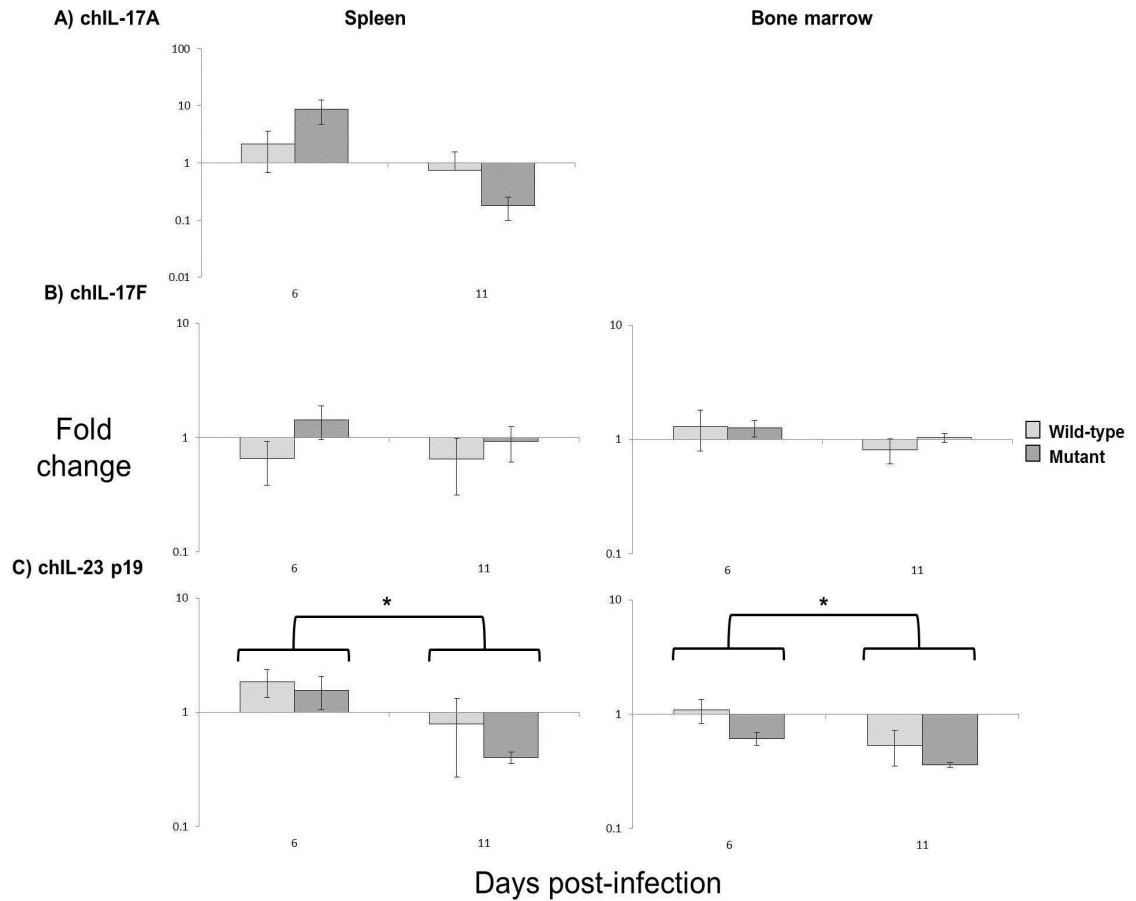


Figure 4.19. Quantification of (A) chIL-17A, (B) chIL-17F and (C) chIL-23 p19 mRNA in the spleen and bone marrow during *S. aureus* infection. Data are expressed as fold change in cytokine mRNA levels of wild-type infected birds (light grey bars) and mutant infected birds (dark grey bars) compared to uninfected age-matched controls \pm SEM at 6 and 11 days post-infection. A 2-way ANOVA with interactions was used to determine if there were significant differences between group and dpi for each cytokine ($p < 0.05$) as indicated by an asterisk. Control values at 6 days are the mean of 2 birds and at 11 days, the mean of 3 birds. Wild-type values at 6 days are the mean of 3 birds and at 11 days are the mean of 4 birds. Mutant values at 6 and 11 days are the mean of 3 birds. Assays were carried out in triplicate.

Chicken IL-17A mRNA expression measured in the bone marrow showed bird-to-bird variation between the groups and dpi (Figure 4.20). Individual birds responded by expressing chIL-17A mRNA or not at all. As no chIL-17A mRNA was measured in the control group at 6 dpi, it was not possible to analyse fold change. With the exception of one bird (WT2), there was higher mRNA expression in the wild-type and mutant groups compared to uninfected controls at 6 dpi. At 11 dpi, there was no difference in chIL-17A mRNA expression between the wild-type, mutant or uninfected control groups.

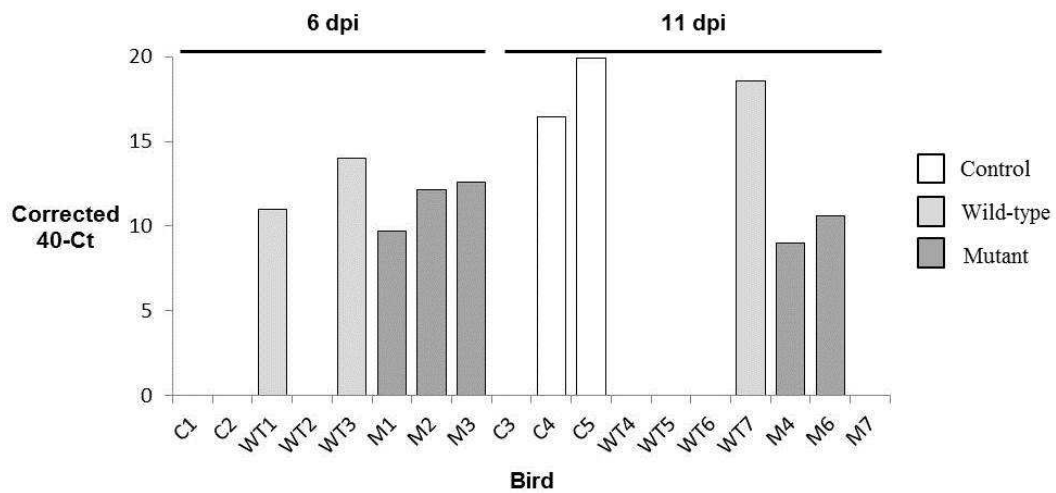


Figure 4.20. Chicken IL-17A mRNA levels in the bone marrow of *S. aureus* infected birds. Data are expressed as corrected 40-Ct cytokine mRNA levels in uninfected control birds (C1-C5) (white bars), wild-type infected birds (WT1-WT7) (light grey bars) and mutant infected birds (M1-M7) (dark grey bars) at 6 and 11 dpi. A Mann-Whitney U test was used to determine if there were significant differences between the wild-type, mutant and uninfected control groups. Assays were carried out in triplicate.

4.4 Discussion

Th17 cells were identified in mammals by Harrington *et al.* (2005) and are characterised by the production of IL-17A, IL-17F, IL-21 and IL-22 (Korn *et al.*, 2009). They are a CD4⁺ T cell subset that have not been described in the chicken to-date despite chickens expressing the characteristic cytokines. Therefore, to study the possible involvement of Th17 immune responses in the chicken, mRNA expression of Th17 cytokines (IL-23, IL-21, IL-17A, IL-17F and IL-22) was measured in various tissues, stimulated cells and during infection by RT-qPCR (TaqMan®). At the beginning of this study, IL-17F had not been positively identified and cloned in the chicken. The gene was incorrectly annotated in the chicken genome. In order to study the expression of Th17 effector cytokines in the chicken, the chIL-17F nt sequence was confirmed. TaqMan® primers and probes were designed against chIL-17A and chIL-17F. This Chapter describes the cloning of chIL-17F and its structural features, as well as measurement of chIL-17A, chIL-17F, chIL-21 and chIL-22 mRNA levels in a wide range of tissues, stimulated cells and during infection in the chicken. Additionally, mRNA expression of chIL-23 p19 was measured during MD and *S. aureus* infection in the chicken to establish if there is also a role for chIL-23 during infection.

4.4.1 Identification of chIL-17F

Chicken IL-17F has high aa identity (39.6%) compared to mammalian IL-17Fs (Kaiser *et al.*, 2005) and is similar in aa sequence length to its mammalian counterparts. Mammalian IL-17A and IL-17F have the highest identity of the IL-17 family of cytokines at approximately 50% (Weaver *et al.*, 2007). This is very similar to chIL-17A and chIL-17F, which share 53% aa sequence identity (Kim *et al.*,

2012b). Mammalian IL-17 family cytokines, in their homodimeric form, display a cysteine knot structure with two disulphide bonds (Liu *et al.*, 2013). This is made up of two pairs of anti-parallel β -strands bundled through two disulphide bonds and a third disulphide bond being replaced by two conserved serine residues (Zhang *et al.*, 2011). The cysteine residues important for disulphide bond formation are conserved in chIL-17F and may be involved in the formation of 2 disulphide bonds. However, only one of the two conserved serine residues is present. Mammalian IL-17A and IL-17F form homodimers (IL-17A/A and IL-17F/F) and a heterodimer (IL-17A/F) (Chang and Dong, 2007). The high aa conservation between chIL-17A and chIL-17F and their mammalian counterparts, particularly within the regions important for secondary structure formation, imply chIL-17A and chIL-17F may also form functional homo- and heterodimers. The positively charged arginine residues that interact with the mammalian IL-17RA (Zhang *et al.*, 2011) are also conserved in the chicken, suggesting chIL-17F may interact with its receptor as in mammals. The cloning of chIL-17F provides an analytical tool for investigating Th17 immune responses in the chicken, and is the first step towards establishing if chIL-17F biological function is similar to mammals.

4.4.2 Chicken IL-17A expression

To begin to elucidate whether chickens mount Th17 immune responses, mRNA expression of the Th17 effector cytokines chIL-17A, chIL-17F, chIL-21 and chIL-22 was measured using RT-qPCR (TaqMan®) in a wide range of tissues and in stimulated splenocytes, thymocytes and bursal cells. Chicken IL-17A was widely expressed in all immune and non-immune tissues measured. These data contradict Min and Lillehoj's (2002) findings where Northern blot analysis of a series of normal

chicken tissues did not detect chIL-17A mRNA. However, more recent findings from Kim *et al.* (2012b) detected chIL-17A mRNA, using RT-qPCR, at low levels in thymus, bursa of Fabricius, small and large intestine, liver, lung, brain and fat but not in spleen, kidney, heart or skin. In Rainbow trout, similarly to chicken, mRNA expression of the IL-17A/F2 molecule was detected in 14 tissues of healthy fish by RT-qPCR (Monte *et al.*, 2013). Early studies in mammals using Northern blot-analysis did not detect IL-17 transcript in normal tissues (Kennedy *et al.*, 1996; Yao *et al.*, 1995b) but pig IL-17A mRNA is detected in heart, skin and jejunum by RT-qPCR (Katoh *et al.*, 2004). These reports suggest that the expression and detection of IL-17A in tissues varies across species but is perhaps more dependent on the detection method used. It is possible that RT-qPCR is a more sensitive detection method than Northern blot analysis used in the published data. Alternatively, chIL-17A mRNA tissue expression may also vary between individual chickens due to breed and age. Min and Lillehoj (2002) used (SPF) white Leghorn chickens (15 weeks old) and Kim *et al.* (2012b) used a Ross line of chicken (10 days old), both of which differ in breed and age from J line birds at 6 weeks old.

Chicken IL-17A mRNA levels were up-regulated in ConA-stimulated splenocytes after six hours. However, over time, chIL-17A mRNA expression declined between 2-24 h post-stimulation. ConA is a polyclonal T cell stimulus, which has similar effects on mammalian and chicken T cells (Stobo, 1972; Toivanen and Toivanen, 1973). Previous reports detected chicken and duck IL-17A expression 24-48 h post-stimulation (Min and Lillehoj, 2002; Yoo *et al.*, 2009). However, both of these avian studies used higher concentrations of ConA to stimulate splenocytes, suggesting higher levels of ConA-stimulation maybe required to measure up-regulation of chIL-

IL-17A mRNA levels in stimulated splenocytes. There was up-regulation of chIL-17A mRNA in stimulated bursal cells but chIL-17A mRNA levels declined between 2-24 h post-stimulation. Chicken IL-17A mRNA levels have not been reported in stimulated thymocytes or bursal cells before this study. In trout, mRNA expression of the IL-17A/F2 molecule was up-regulated by several mitogens including PHA and PMA and calcium ionophore in the head kidney, a major lymphoid organ in the fish (Monte *et al.*, 2013). However, the specific cell types expressing the IL-17A/F2 molecule were not investigated. In humans, stimulated B cells express IL-17A (Vazquez-Tello *et al.*, 2012). The majority (98%) of bursal lymphocytes are B cells (Schat *et al.*, 2012) so it is unsurprising that stimulated bursal cells up-regulate chIL-17A mRNA expression.

Measuring chIL-17A mRNA in stimulated primary cells is the first step to investigate how IL-17A expression in the chicken compares to mammals. To further this study, investigation of appropriate polyclonal stimuli that drive chIL-17A expression in stimulated cells, and at what concentrations, would be useful as a possible indicator of what drives chIL-17A expression *in vivo*. In humans, IL-17A is expressed by CD4⁺ and CD8⁺ T cells, $\gamma\delta$ T cells, NKT cells and ILC3's (Reynolds *et al.*, 2010). It would be interesting to investigate the biological and immunological properties of chIL-17A by comparing the cells expressing IL-17A in the chicken and mammals. There are mouse Abs to chicken CD4⁺, CD8⁺ and $\gamma\delta$ T cells (Davidson and Boyd, 1992; Sowder *et al.*, 1988), which could be used with an Ab to rchIL-17 (Yoo *et al.*, 2008) to investigate the cells that express IL-17A in the chicken.

4.4.3 Chicken IL-17F expression

The expression of chIL-17F mRNA was ubiquitous in the range of tissues examined. Chicken IL-17F mRNA levels were lower and more variable in the tissues measured compared to chIL-17A. These data correlate with previous studies in the chicken, where chIL-17F was detected widely in tissues but at low levels (Kim *et al.*, 2012b), and with studies in fish, where the IL-17A/F2 molecule was detected in all tissues examined (Monte *et al.*, 2013). In mammals, IL-17A and IL-17F have similar structures and properties and signal through the same receptors (Toy *et al.*, 2006; Weaver *et al.*, 2007; Wright *et al.*, 2008). However, the distinct functions of IL-17A and IL-17F, particularly in mucosal infection and autoimmune disease, have only begun to be elucidated (Ishigame *et al.*, 2009; Iwakura *et al.*, 2011). Further investigation of the possible overlapping and distinct cellular expression, and biological functions of chIL-17A and chIL-17F is needed, to establish similar roles to mammals.

Chicken IL-17F mRNA levels were up-regulated compared to unstimulated controls in ConA-stimulated splenocytes. There was no relationship in chIL-17F mRNA levels over time. This contrasts with recently published data showing chIL-17F mRNA to generally increase in a time-dependent manner, peaking after 24 h, following mitogen stimulation of splenocytes (Kim *et al.*, 2012b). This could be due to the variation in chIL-17F mRNA expression found in different chicken lines and tissues sampled (Kim *et al.*, 2012b). There was no difference in chIL-17F mRNA levels between control and stimulated cells in the thymus suggesting PHA-stimulation of thymocytes does not increase chIL-17F mRNA expression.

Chicken IL-17F mRNA in stimulated bursal cells was up-regulated but overall chIL-17F mRNA levels decreased until 18 h post-stimulation. These data suggest chicken B cells are capable of expressing and increasing expression of chIL-17F upon stimulation, similarly to human B lymphocytes (Vazquez-Tello *et al.*, 2012), despite human IL-17F mainly being expressed by CD4⁺ and CD8⁺ T cells, $\gamma\delta$ T cells, epithelial cells and other innate cells (Reynolds *et al.*, 2010). To-date, there is no chIL-17F Ab but the development of one would be useful to further investigate the cells that express chIL-17F. Further molecular tools are required to shed light on the biological function of chIL-17F, both in relation to chIL-17A function and within Th17 immune responses in the chicken.

4.4.4 Chicken IL-21 expression

Chicken IL-21 mRNA was widely expressed in a range of tissues at low levels. However, chIL-21 mRNA was not detected in muscle or skin. IL-21 tissue expression has been investigated in cattle and fish (Muneta *et al.*, 2003; Wang *et al.*, 2011). Bovine IL-21 was only detected in the spleen by RT-PCR (Muneta *et al.*, 2003), in contrast to fish, where IL-21 was measured by RT-qPCR in all tissues examined (Wang *et al.*, 2011). Similarly to fish, higher chIL-21 mRNA levels were seen in the gut compared to other non-lymphoid tissues, probably due to the high levels of lymphoid cells found in this tissue. In SPF birds, the highest levels of chIL-21 mRNA were detected in lymphoid tissues rich in plasma cells, specifically Meckel's diverticulum, the spleen and the Harderian gland (Rothwell *et al.*, 2012), which is corroborated here.

Chicken IL-21 mRNA levels in stimulated splenocytes were up-regulated, with chIL-21 mRNA expression increasing over time. In chickens, IL-21 is expressed by CD4⁺

and TCR $\alpha\beta^+$ cells and expression is up-regulated by mitogen stimulation (Rothwell *et al.*, 2012). In mammals, IL-21 is expressed by CD4 $^+$ T cells and NKT cells (Coquet *et al.*, 2007; Parrish-Novak *et al.*, 2000). The increased chIL-21 mRNA levels in splenocytes compared to unstimulated controls may represent induction of chIL-21 mRNA in these cell types.

Stimulated bursal cells increased expression of chIL-21 mRNA from 2-6 h and then chIL-21 mRNA levels decreased between 6-24 h post-stimulation. It is unclear why increased expression of chIL-21 mRNA was detected in stimulated bursal cells. The bursa of Fabricius is 98% B cells (Schat *et al.*, 2012) and in SPF birds, B cells stimulated with PMA expressed higher levels of chIL-21 mRNA compared to unstimulated controls (Rothwell *et al.*, 2012). However, the low levels detected were attributed to contaminating CD4 $^+$ T cells. The chIL-21 mRNA levels measured in stimulated bursal cells in this study are likely to be too high to be justified by contamination of T cells. Therefore, contrary to mammals, these data suggest that chicken B cells when stimulated with PMA and ionomycin express increased levels of chIL-21 mRNA.

4.4.5 Chicken IL-22 expression

Expression of chIL-22 mRNA was measured in various tissues using RT-qPCR (TaqMan®) and was detected at very low levels in all tissues. Chicken IL-22 mRNA was also measured in stimulated lymphoid tissues and compared to unstimulated controls. There was no up- or down-regulation of chIL-22 mRNA expression, or relationship over time, in any of the stimulated cells. Previous studies in mammals have shown that mitogen-activated human and mouse $\alpha\beta$ T cells, NK cells and monocytes express IL-22 (Dumoutier *et al.*, 2001; Wolk and Sabat, 2006; Xie *et al.*,

2000). Chicken IL-22 mRNA expression from these cells types would explain the low detection of chIL-22 mRNA in the spleen but not in the bursa of Fabricius. Mammalian IL-22 expression in B cells has not been reported. Mammalian IL-22 is primarily expressed by activated Th17 cells (Liang *et al.*, 2006). The thymus is predominantly made up of developing and naïve T cells, which these data suggest are not capable of expressing chIL-22 mRNA.

To summarise, all chicken Th17 effector cytokines were constitutively expressed in normal tissues. Chicken IL-17F, chIL-21 and chIL-22 mRNA levels were up-regulated in stimulated splenocytes and bursal cells, with higher expression compared to unstimulated controls seen at earlier time-points, but chIL-17A mRNA was not. Th17 effector cytokine mRNA expression in different lymphoid cells of the chicken can be modulated *in vitro* by a range of stimulants. It would be interesting to investigate the cell types in the chicken expressing Th17 effector cytokines to further compare and contrast avian Th17 immune responses with mammals. Identification of the cell types which express the Th17 effector cytokines may also explain why it was not possible to induce expression of chIL-17A, chIL-17F, chIL-21 or chIL-22 in stimulated thymocytes.

4.4.6 Infection models

In addition to investigating whether mRNA expression of Th17 effector cytokines could be modulated in primary cells in the chicken, further investigation of whether chickens mount Th17 immune responses was carried out by measuring mRNA of chIL-17A, chIL-17F, chIL-21 and chIL-22 during MD, Infectious bursal disease and histomoniasis infection. MD and Infectious bursal disease are recognised as being controlled by Th1 immune responses (Liu *et al.*, 2010; Xing and Schat, 2000)

whereas histomoniasis is controlled by Th2 immune responses (Powell *et al.*, 2009). Messenger RNA expression of chIL-17A, chIL-17F and chIL-23 were also measured during *S. aureus* infection of chickens. In mammals, *S. aureus* induces Th17 immune responses (Cho *et al.*, 2010) and is already established as a disease model in poultry (Daum *et al.*, 1990). Archived RNA samples from previous *in vivo* challenge experiments were examined, with the exception of *S. aureus*. Tissues which expressed higher mRNA levels of Th1/Th2 effector cytokines during the course of infection were selected for measurement of Th17 effector cytokine expression. Sampling time points were restricted by the original experiment and were selected on availability of sample.

4.4.6.1 Marek's disease

The mRNA levels of the Th17 effector cytokines and IL-23 p19 were measured in the spleens of MDV-infected chickens. Chicken IL-23 p19, chIL-17A and chIL-21 mRNA levels were decreased in infected resistant line 6₁ chickens and increased in infected susceptible line 7₂ chickens compared to uninfected controls. Chicken IL-17A is a pro-inflammatory cytokine and its elevated expression early in cytolytic infection may simply represent a response to increased pathology in susceptible birds. However, the early involvement of IL-17 immune responses appears to be associated with susceptibility to MDV infection. Resistance in line 6₁ birds has been mapped to a genetic locus outside of the MHC (MDV-1) (Bumstead, 1998). It has been postulated this resistance resides at the level of target cell number and their susceptibility to MDV (evident during the lytic phase) (Lee *et al.*, 1981). The apparent reduction in Th17 immune responses in resistant line 6₁ birds early in MDV infection raises the question is the reduction in Th17 cytokine expression in infected

resistant birds because of reduced pathology or does it potentially effect the numbers of activated B and T cell numbers during MDV-infection?

Chicken IL-17A mRNA levels were higher in MDV-infected birds compared to mRNA levels in age-matched, uninfected controls at 3 and 4 dpi, irrespective of the line of bird. These data suggest innate levels of chIL-17A increase over time from the point of MDV-infection, regardless of resistance or susceptibility to MD. Further study is required to investigate if, or indeed when, this IL-17 response declines and whether MDV strain alters this response. The early differences in chIL-17A, chIL-21 and chIL-23 p19 mRNA levels between susceptible and resistant genotypes indicate these cytokines may play a role in adaptive immune responses that in susceptible lines result in tumour formation and in resistant lines result in lesion regression.

Chicken IL-17F mRNA expression was decreased in MDV-infected chickens compared to uninfected chickens irrespective of the line of bird or the time point measured. Interestingly, chIL-17A and chIL-17F mRNA levels demonstrated differential expression patterns during MDV-infection. Chicken IL-17A mRNA levels were dependent on the line of bird and the dpi but chIL-17F mRNA expression was dependent on MDV-infection. As discussed previously, the distinct biological functions of IL-17A and IL-17F in mammals are not clear. The data here suggests potentially different roles for chIL-17A and chIL-17F during MDV infection.

IFN- γ expression is up-regulated during MDV infection at 3-10 dpi in both susceptible and resistant lines of chicken, associated with increasing MDV loads (Kaiser *et al.*, 2003). IFN- γ is expressed by Th17 cells in mammals but also acts to inhibit Th17 immune responses, depending on the context and amount of IFN- γ

expressed (Harrington *et al.*, 2005; Hirota *et al.*, 2011). Therefore, expression of the Th17 effector cytokines and IL-23 in MDV-infected chickens after 3 dpi needs further study to investigate if chIFN- γ expression alters Th17 effector cytokine expression. The role of Th17 cytokines in MDV pathogenesis remains unclear and further investigation over a longer time course is needed to fully elucidate the complex adaptive immune responses mounted in response to MDV infection.

4.4.6.2 Infectious bursal disease

Messenger RNA levels of the Th17 effector cytokines were also measured during IBDV infection. IBDV targets the chicken immune system in a complex and poorly understood manner (Ingrao *et al.*, 2013). The molecular basis of pathogenicity is unclear, however, new studies highlight the role of an exacerbated innate immune response during early infection, up-regulating pro-inflammatory mediators and inducing a cytokine storm (Rauw *et al.*, 2007). The expression of Th17 cytokines during IBDV infection has not been reported to-date. Interestingly, chIL-17A and chIL-17F mRNA levels were differentially expressed during the acute phase of IBDV infection. At 1 dpi, chIL-17A mRNA levels were up-regulated and chIL-17F mRNA levels reduced. By 3 dpi, chIL-17A mRNA levels were down-regulated, and the reverse was true of chIL-17F mRNA levels, which were increased. As previously discussed, the distinct expression of mammalian IL-17A and IL-17F during disease is only just beginning to be revealed (Dubin and Kolis, 2009); why chIL-17A and chIL-17F mRNA expression is different in the early stages of IBDV infection needs to be investigated further. Chicken IL-21 mRNA levels were reduced at later time points, whereas chIL-22 mRNA levels were up-regulated. Overall, all Th17 cytokine

mRNA levels detected were low, suggesting these cytokines are not induced as part of a cytokine storm during the innate response to IBDV infection.

This study measured Th17 cytokines in caecal tonsil from SPF birds infected with a very virulent IBDV (vvIBDV) strain. Rauw *et al.* (2007) reported high levels of systemic $\text{chIFN-}\gamma$ during the acute phase of vvIBDV infection. Despite reports of Th17 cells able to express IFN- γ (Lee *et al.*, 2009), high levels of IFN- γ inhibit Th17 immune responses (Villarino *et al.*, 2010; Yeh *et al.*, 2014). IFN- γ is up-regulated during IBDV infection (Rautenschlein *et al.*, 2002; Ruby *et al.*, 2006), which could explain the low mRNA levels of Th17 cytokines measured. Other cytokines shown to be up-regulated during IBDV infection are the pro-inflammatory cytokines IL-2 (Rautenschlein *et al.*, 2002), IL-18 and IL-6 (Ruby *et al.*, 2006), suggesting activation of T cells, particularly Th1 cells. IL-2 and Th1 immune responses also inhibit Th17 immune responses (Laurence *et al.*, 2007; Lazarevic *et al.*, 2011). Kim *et al.* (1998) reported the down-regulation of TGF- β 4 during IBDV infection. TGF- β 4 is the avian homologue of mammalian TGF- β (Jakowlew *et al.*, 1997), which is required for Th17 cell differentiation (Veldhoen *et al.*, 2006), further suggesting the inhibition of Th17 immune responses during IBDV infection. The possible involvement of Th17 immune responses during both the early and the later stages of IBDV infection in the chicken require further investigation.

4.4.6.3 Histomoniasis

The mRNA levels of Th17 effector cytokines were also measured in the caecal tonsil during *H. meleagridis* infection in the chicken. All cytokine mRNA levels were initially up-regulated. Chicken IL-17A and chIL-21 mRNA levels decreased over

time until 12 dpi and then increased from 18-30 dpi. Messenger RNA levels of chIL-17F and chIL-22 decreased over the time course.

The original study by Powell *et al.* (2009) demonstrated increased expression of chIL-1 β , chIL-6 and chTGF- β 4 and low levels of chIFN- γ , chIL-13 and chIL-4 early in infection in broiler chickens. The increased expression of IL-1 β drives expression of IL-17A and IL-22 from innate cells during early infection in mammals (Coccia *et al.*, 2012), possibly explaining the up-regulated mRNA levels of chIL-17A and chIL-22 cytokines measured initially. It may be that the release of chIL-17A is important in the initial innate immune responses in the caecal tonsil to limit movement of protozoa to the liver. The innate immune response is activated immediately upon encountering a pathogen and continues for approximately 4 days, at which point infection is either effectively overcome or the adaptive immune response is triggered. All Th17 effector cytokine mRNA levels decreased up to 4 dpi, suggesting a decline in innate immune responses. By 5 dpi, there was increased mRNA expression of chIFN- γ as well as up-regulation of chIL-13, a signature Th2 cytokine (Powell *et al.*, 2009). Th1 and Th2 cytokines inhibit Th17 immune responses in mammals (Cooney *et al.*, 2011; Lazarevic *et al.*, 2011) so may explain the continued decreasing levels of Th17 cytokine mRNA levels seen at this time. From 12 dpi, there was evidence of caecal healing occurring at a gross level and sustained elevation of chIL-13 mRNA levels (Powell *et al.*, 2009). At this time, all Th17 effector cytokine mRNA levels were down-regulated, possibly due to elevated Th2 cytokine expression. The reduction in gross pathology in the caeca may be attributed to the down-regulation of the pro-inflammatory cytokines chIL-17A and chIL-17F. The importance of IL-17 in

the context of gut inflammation is highlighted by the expression of IL-17A and IL-17F in humans with IBD (Fujino *et al.*, 2003).

Two Th17 effector cytokines, chIL-17A and chIL-21, showed a decline and then an increase in mRNA levels whereas chIL-17F and chIL-22 mRNA expression decreased over time. Why chIL-17A and chIL-21 mRNA showed different expression patterns to chIL-17F and chIL-22 during *H. meleagridis* infection is unclear. IL-17A is the signature cytokine of Th17 immune responses (Aggarwal *et al.*, 2003), and by the recruitment of neutrophils, is involved in overcoming infection with extracellular pathogens in mammals. The relationship of chIL-17A mRNA expression with time suggests involvement of IL-17 immune responses during infection with the extracellular pathogen *H. meleagridis*. Therefore, chIL-17A and chIL-23 p19 mRNA levels in additional tissues, and more time points between 12 and 30 dpi, should be investigated to elucidate the extent of Th17 involvement in the immune response to *H. meleagridis* infection.

4.4.6.4 *Staphylococcus aureus*

Finally, mRNA levels of chIL-17A, chIL-17F and chIL-23 p19 were measured in a *S. aureus* infection model in the chicken. Measurement of these cytokines was based on studies in mammals. IL-17A and IL-17F-deficient mice are more susceptible to opportunistic infections with *S. aureus* (Ishigame *et al.*, 2009) and *S. aureus* peptidoglycan preferentially induces IL-23 expression (Smits *et al.*, 2004). BCO pathogenesis requires haematogenously distributed bacteria and lesions are most commonly found associated with the growth plates of the long bones, particularly the femur (McNamee and Smyth, 2000; Wideman and Prisby, 2012). Therefore, the

spleen and bone marrow of the femur were sampled for cytokine mRNA measurement. This was a pilot study to identify an optimal infection dosage of *S. aureus*, which would allow measurement of disease symptoms for the longest possible time course. All three cytokines were detected in the spleen and bone marrow at all time-points. There was no difference in chIL-17A mRNA expression in the spleen or chIL-17F mRNA expression in the spleen and bone marrow. However, chIL-17A mRNA expression in the bone marrow varied from bird-to-bird within the groups. There was higher mRNA expression of chIL-17A in the wild-type and mutant groups at 6 dpi, suggesting the possibility of a local chIL-17A response to *S. aureus* infection.

During the study, the birds appeared to be overcoming the initial infection. Therefore, half of the birds from each group were culled and sampled after 6 dpi, and the remainder sampled at 11 dpi, to detect any changes in cytokine mRNA levels between infected and recovered birds. As the only variation in cytokine mRNA levels between infected and control groups was seen in chIL-17A mRNA expression in the bone marrow, it is possible cytokine levels were already returning to normal levels following resolution of the *S. aureus* infection. The bird-to-bird variation in chIL-17A mRNA levels in the bone marrow requires further investigation and may suggest early local involvement of IL-17A in response to *S. aureus* infection. Because of the lack of response detected in the tissues sampled and cytokines measured, it was decided not to investigate mRNA levels in the additional tissues sampled or, indeed of the other Th17 effector cytokines, chIL-21 and chIL-22.

The *S. aureus* study was a pilot study and due to time constraints, further investigation of Th17 immune responses during *S. aureus* infection of the chicken

was not possible. The apparent recovery from *S. aureus* infection suggests a higher infective dose of bacteria is required to establish BCO in this breed of chicken. Also, a time course over which to sample infected birds would hopefully highlight any relationship between chIL-17A, chIL-17F and chIL-23 p19 mRNA expression and *S. aureus* infection. Measuring cytokine expression in additional lymphoid and non-lymphoid tissues such as the bursa of Fabricius, thymus and liver may also indicate any systemic involvement of *S. aureus* during BCO.

4.4.7 Summary

The aims of this chapter were to investigate Th17 immune responses in the chicken by measuring mRNA levels of Th17 effector cytokines and IL-23 in various tissues, cells and during infection of the chicken and compare to mammals. The development of Th17 reagents in the chicken, such as chIL-17A and chIL-17F TaqMan® primers and probes, permit investigation of how and where Th17 immune response components are expressed and their potential role during infection. Th17 mRNA levels were detected in nearly all tissues sampled although at low levels. The cytokine most highly expressed in tissues was chIL-17A. Stimulated lymphoid cells generally up-regulated expression of chicken Th17 effector cytokine mRNA, with levels decreasing over time. These data warrant further investigation of the specific cell types expressing the Th17 cytokines in these lymphoid tissues. The infection model experiments used archived samples, or were initial pilot studies, and investigation of Th17 effector cytokine levels, using additional tissue samples and longer time course studies, is required to fully investigate the role of Th17 immune responses in the chicken. However, confirmation that chicken Th17 effector

cytokines are expressed is highly suggestive of the involvement of Th17 immune responses in the bird.

Chapter 5 Identifying the chIL-23R

5.1 Introduction

T helper cells are functionally distinguished on their patterns of cytokine secretion (Mosmann *et al.*, 1986). Th1 cells express high levels of IFN γ , Th2 cells express IL-4 and Th17 cells predominantly express IL-17A (Brucklacher-Waldert *et al.*, 2009). Additionally, Th cells can be phenotypically distinguished by the expression of cell surface markers and transcription factors. The master regulator for transcription of Th1 cells is T-bet and for Th2 cells is GATA3 (Rengarajan *et al.*, 2000). Th17 cells universally express IL-23R (McGeachy *et al.*, 2009), RORC, ROR γ t (Ivanov *et al.*, 2006) and CCR6 (Acosta-Rodriguez *et al.*, 2007b), and can additionally express a number of chemokine receptors to regulate the migration of Th17 cells to peripheral tissues (Lim *et al.*, 2008). As a result, mammalian Th17 cells are primarily identified by expression of these molecules, using antibodies for intracellular staining of IL-17A in CD4⁺ T cells and detecting cell surface expression of IL-23R (Harrington *et al.*, 2005; Veldhoen *et al.*, 2006; Wilson *et al.*, 2007). However, due to the low expression of IL-23R (Chognard *et al.*, 2014) and a lack of appropriate antibody reagents to identify and track IL-23R-bearing cells *in vivo* (Awasthi *et al.*, 2009), both knock-in and knock-out IL-23R-reporter mice have been developed to investigate mammalian IL-23R (Awasthi *et al.*, 2009).

In mammals, the generation of normal Th17 cell effector responses requires IL-23R-dependent signalling (McGeachy *et al.*, 2009) as IL-23 is required for the stabilisation of the Th17 phenotype during an immune response. IL-23R is a member of the haemopoietin receptor superfamily and is made up of two subunits, IL-23R

and IL-12R β 1 (Parham *et al.*, 2002). In humans, IL-23R is expressed on activated and memory T cells as well as $\gamma\delta$ and CD8 T cells and B cells. It is also expressed by innate lymphoid cells (Chognard *et al.*, 2014; Parham *et al.*, 2002). The role of the IL-23R in cell-mediated inflammatory processes in conjunction with IL-23, during autoimmune diseases, as well as in the pathogenesis of many cancers, is well-established in mammals (Cargill *et al.*, 2007; Duerr *et al.*, 2006; Yao *et al.*, 2014). Therefore, genetic polymorphisms of the mammalian IL-23 pathway are strongly associated with susceptibility to inflammatory disease (Duvallet *et al.*, 2011). There are 24 splice variants of the human IL-23R, suggesting the potential for considerable biological diversity in the IL-23R gene (Kan *et al.*, 2008; Zhang *et al.*, 2006).

The IL-23R has been successfully identified in the pig (Kokuho *et al.*, 2012) and cattle (Chen *et al.*, 2009) but not yet in the chicken. Three single nucleotide polymorphisms (SNP) have been identified in cattle, with the potential to alter gene expression and protein function but further study is required with respect to their biological and practical relevance (Skelding *et al.*, 2010).

The identification of IL-23 and its receptor in mammals initiated the discovery of a third Th subset, Th17, which is yet to be confirmed in the chicken. Chicken IL-23 p19 has not been identified in the chicken genome (Chapter 3) and the chIL-23R is misannotated in the current chicken genome build. To enable biological analysis of the chIL-23R, similarly to chIL-23 p19, basic genetic information about the chIL-23R molecule is required. The aims of this Chapter were to identify and clone the IL-23R in the chicken and investigate the structural similarities and differences between avian and mammalian IL-23R. Further, to measure mRNA expression of the chIL-23R in tissues and stimulated cells. The longer term goal of this study was to be able

to positively identify Th17 cells in the chicken and, therefore, confirm the presence of Th17 immune responses in birds.

5.2 Methods

General methods are outlined in Chapter 2. Additional methods and alterations are detailed here.

5.2.1 Cloning of the *chIL-23R*

At the beginning of this study, the *chIL-23R* cDNA sequence was incorrectly annotated in the chicken genome (WASHUC2, Ensembl). The annotated sequence for the *chIL-23R* in Ensembl (ENSGALT00000018285) appeared to be missing several exons (Figure 5.1), suggesting the correct *chIL-23R* sequence to be the GenScan prediction (GENSCAN00000023581) located on the same chromosome as the misannotated *chIL-23R* gene and the annotated *chIL-12R β 2* gene.

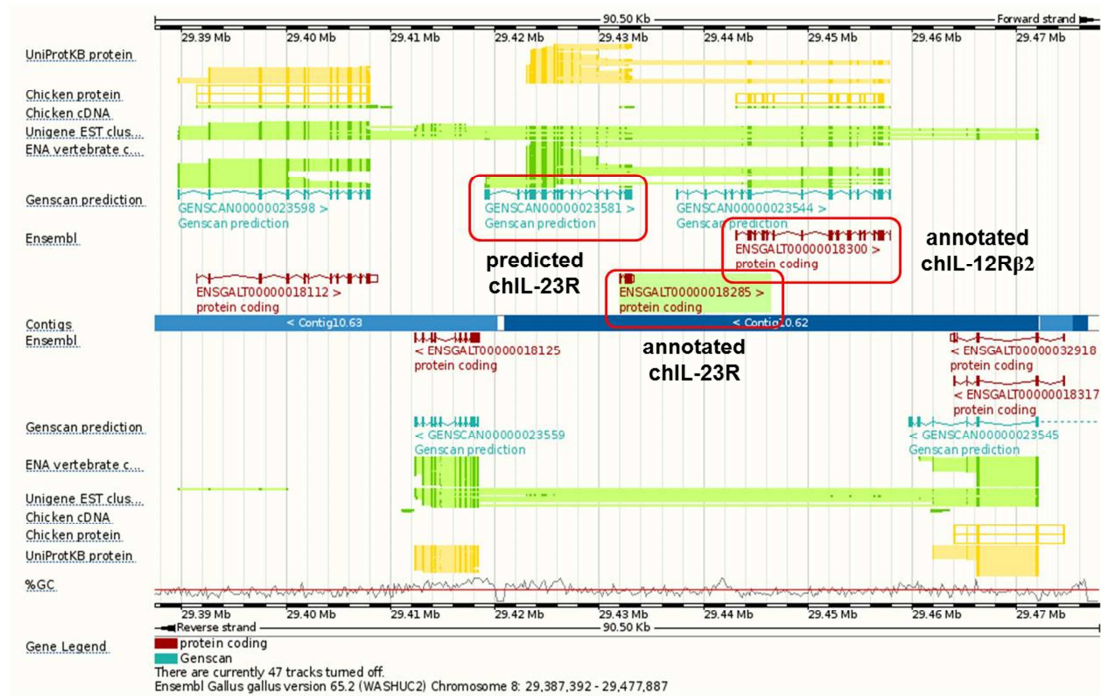


Figure 5.1. Ensembl (WASHUC2) showing the genetic location of the chIL-23R (GENSCAN00000023581) on chromosome 8 of the chicken genome. Red boxes highlight the predicted chIL-23R (GENSCAN00000023581), and the annotated genes for chIL-23R (ENSGALT00000018285) and chIL-12R β 2 (ENSGALT00000018300), located downstream on the same chromosome.

Using the GenScan prediction for the chIL-23R (GENSCAN00000023581) (Figure 5.1), multiple forward and reverse primer sets (Appendix 5) were designed to amplify the full-length chIL-23R, of varying lengths and GC content, as well as covering different parts of the predicted sequence. In addition to multiple primer sets, oligo(dT)₂₀ and gene-specific reverse primers were used to generate cDNA from RNA extracted from chicken splenocytes stimulated with mitogen (ConA and PHA). These cDNA templates were then used in subsequent PCR reactions, using all possible combinations of forward and reverse primers, as well as testing different annealing temperatures and extension times for the various primer combinations. Optimisation of the GC-Rich PCR System (Roche), which allows for improved amplification of sequence with a high GC content, using primers IL-23R_F10 and

IL-23R_R1b (Table 2.1), increased amounts of RNA in cDNA synthesis and a high MgCl_2 concentration, amplified the full-length sequence of the chIL-23R. The chIL-23R cDNA clone was TA-cloned into pGEM®-T Easy and sequenced. Bioinformatic characterisation of the chIL-23R nt and aa sequences was carried out to confirm the identity of chIL-23R and similarity to mammalian IL-23R sequences.

5.2.2 RT-qPCR (TaqMan®) primer and probe design

Having confirmed the full-length nt sequence of the chIL-23R, primers and probe for use in RT-qPCR (TaqMan®) were designed using Primer Express v3 (Applied Biosystems) to detect chIL-23R mRNA expression (Table 2.4).

5.3 Results

5.3.1 Identification and cloning of the chIL-23R

Based on the predicted chIL-23R nt sequence (GENSCAN00000023581), primers IL-23R_F10 and IL-23R_R1b (Table 2.1) were designed to clone cDNA encoding the predicted full-length chIL-23R, using cDNA prepared from ConA-stimulated splenocytes as a template. Figure 5.2 shows the product amplified with primers IL-23R_F10 and IL-23R_R1b. It is approximately 2.5 kb nt in size and was TA-cloned into pGEM®-T Easy and transformed into *E.coli* JM109 competent cells for subsequent plasmid isolation and sequencing.

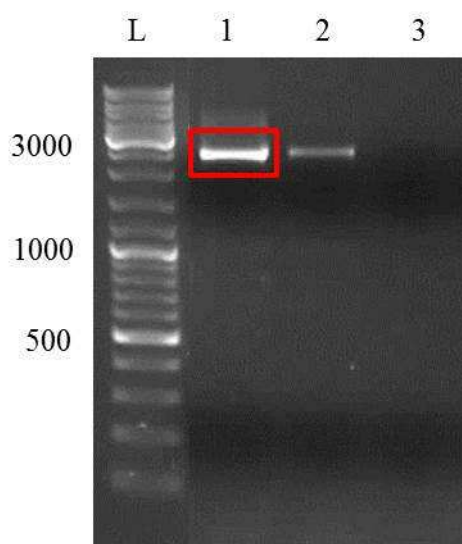


Figure 5.2. RT-qPCR amplification of the predicted chIL-23R. Lane L: GeneRuler™ DNA Ladder Mix (Thermo Scientific). Lane 1: cDNA from ConA-stimulated (2 h) splenocytes. Lane 2: cDNA from ConA-stimulated (4 h) splenocytes. Lane 3: negative control. Red box highlights PCR product selected for TA cloning and sequencing.

Multiple cloned plasmids were sequenced to confirm the nt sequence of the chIL-23R, which also confirmed the misannotation of the chIL-23R gene (ENSGALT00000018285) in the Ensembl database (WASHUC2). Further bioinformatic characterisation of the chIL-23R nt and aa sequences was carried out to confirm the gene's similarity to mammalian IL-23R sequences.

5.3.2 Structural features of the *chIL-23R*

The full-length *chIL-23R* nt sequence was translated and the aa sequence further characterised *in silico*. The *chIL-23R* encodes a predicted protein of 816 aa, with a putative 55 aa signal peptide (Figure 5.3). The human IL-23R cDNA encodes a predicted protein of 629 aa, with a putative 23 aa signal peptide, resulting in a predicted protein of 606 aa. When aligned with mammalian IL-23R sequences, the *chIL-23R* has an identity of 27.1% at the predicted aa level compared to the human, mouse, pig and cattle IL-23Rs. The *chIL-23R* aa sequence is much longer than the mammalian IL-23R sequences. Further, there is 33.5%, 31.6%, 32.8% and 32.7% aa similarity between the *chIL-23R* and human, mouse, pig and cattle IL-23Rs respectively. There are 19 cysteine residues in the *chIL-23R*, 11 of which are conserved between the chicken and mammals. There are 11 N-linked glycosylation sites in the *chIL-23R*. Two of these are conserved in all the mammalian IL-23R's and one is conserved in just the mouse IL-23R. The transmembrane domain of the *chIL-23R* contains a sequence (WSAWG) similar to the cytokine receptor signature WSXWS-like motif, which is characteristic of the mammalian type I cytokine receptor superfamily. In the intracellular domain of the *chIL-23R*, there are 6 tyrosine residues, only one of which is conserved (Tyr⁷¹⁸) between the chicken and mammals.

```

chicken : MNAFTVLEANEVSAHIPKAMPHCPTQAAAAAQAAMVGGGEAFVLHLLCIL : 50
human   : -----MNQVTIQWDAVIALYILFSWC : 21
mouse   : -----MKREREMRGFYIWDMSHLTLQLHVIALYVLEFRC : 36
pig     : -----MNQVTTQWDVVIALYIFENWC : 21
cattle  : -----MNQVTIHWDVVIALYIIFSWC : 21

      ↓ ↓
chicken : GRGAAQIRCLGHVRIEFGSVVPMGNSVSIITCVSALG-CPWASLSILLNLT : 99
human   : HGGITNINCSGHIWVEPATIEFMGMNISIYCOAAIKNCQPRKLHFKNGI : 71
mouse   : HGGITSINCSGDMWVEPGEIEFMGMNVIYCOEALKHCRPRNLYFYKNGF : 86
pig     : HGGITNINCSGHIWVEPAAVEFMGMNISIYCOATIKNCQPRKIYFYKNGI : 71
cattle  : HGGITNINCSGHIWVEPATIEFMGMNISIYCOAAIKNCQPSKLYFYKNGI : 71
          * * *

chicken : APKDPPRPINSSAAQLQLRGTRLEFSITACLARCPNSYWNEVVCGETLWA : 149
human   : KERFQITRINKTARLWYKNFLEPHASMYCTAECPKHFQETLICGKDISS : 121
mouse   : KEEDITRINRTARIWYKGFSEPHAYMHCTAECPGHFQETLICGKDISS : 136
pig     : KERFQITRINKTISRLWYNNFLEPQAFVYCTAECPGYFQETLICGKDISS : 121
cattle  : KERFHITRINKTARLWYNNFVEPQAFVYCTAECSTRYFPETLICGKDISS : 121
          * * *

chicken : GYPPLGLPTNLSAIPERSCSLACTWDAGQPTLLHTNYSLHMRSIQTAQED : 199
human   : GYPDPDIPDEVTCVIYEYSCNMTCTWNAAGKLTIDTKYVVHVKSLETEEEQ : 171
mouse   : GHPPDAPSNLTCVIYEYSCNMTCTWNTGKPTYIDTKYIVHVKSLETEEEQ : 186
pig     : GYPDPVDPKVTCTVIYEYSCNMTCTWDAGKPTYIATKYVVYVKSLETEEEQ : 171
cattle  : GYPDPVDPKVACVIYEYSCNMTCTWNPGRPTYIDTKYVVYVKSLETEEEQ : 171
          * *

chicken : VFPTGSPVPLS--ALNGSSRYLAWVOASNALGMARSAPLHLDLQQLVSPA : 247
human   : QYLTSSYINISTDSLQGGKKYLWVVOAANALGMEESKQLQIHLDLDIVIPS : 221
mouse   : QYLASSYVKISTDSLQGSRKYLWVVOAVNSLGMENSQQLHVHLDLDIVIPS : 236
pig     : EYLTSSYINISTDSLQGSKKYLWVVOATNVLGMEKSKQLQVHLDLDIVIPS : 221
cattle  : EYLTSSYINISTDSLQGGKKYLWVVOASNVLGMEKSKQLQIHLDLDIVIPS : 221

chicken : VPLAESAEITAGAPPTTIRWRQOTELQDVRCEERHKAVGAAEWHVTAWD : 297
human   : AAVISRAETINATVPKTIYWDQSOTLIEKVSCEMRYKATTNQTWNVKEFD : 271
mouse   : ASIISRAETINDTVPKTIYVWKSMTIEKVFCEMRYKTTNQTWTSVKEFD : 286
pig     : ASIISRAEDINTTVPRTVIHWSSQTSIERVSCEMRYKATTNQTWKVKEFD : 271
cattle  : ASIISRAEDINTTVPKTVIHWDSQTSIEKVSCEMRYKDTTNQTWNVKEFD : 271
          *

chicken : S---AVQRGHQLQSATRYVFOARCRISAAG----- : 324
human   : TNFTVQQSEFYLEPNIKYVFQVRCQETG-----K : 301
mouse   : ANFTVQQSEFYLEPDSKYVFQVRCQETGKRNWQPWSSPFVHQTSQETGK : 336
pig     : VNLTVMQQSEFYLOPNTKYIFQVRCQETG-----K : 301
cattle  : TNFTVQQSEFYLOPNAMYVFQVRCQETG-----K : 301
          *

chicken : SPWSAAGLPFTYSTPEAAFAAAPDVWRRRLGRLFFNGSHEVTVLIKPPRDA : 374
human   : RYWPWSSPFFHKTPETVPQVTSKAE----- : 327
mouse   : RNWPWSSPFFVHQTSTQVTSQVTAKS----- : 361
pig     : RYWPWSSPFFHKTPETVPQVTVKSE----- : 327
cattle  : KYWPWSSPFFHKTPETVPQVTMKSE----- : 327

chicken : RTPILGYAVWAGASRPLONTSSSTACSLLLPPALRDLRVTAYNARGASSPA : 424
human   : ----- : -
mouse   : ----- : -
pig     : ----- : -
cattle  : ----- : -

```

For figure legend, see page 180

chicken	: RVTLRDDPSPEAFPSPLAVDVQRENQSTVSVAVQLPRHNGTPPLWFIVEW	: 474
human	: -----	: -
mouse	: -----	: -
pig	: -----	: -
cattle	: -----	: -

chicken	: VCTAPYSHEEEFFWKVPGQDSHTYIQEDAAAGSHINVSVAVYPNGVSK	: 524
human	: -----QHDTWNNSGTLTVASISTGELTSDNR	: 351
mouse	: -----SHEPQKMEMLSATIFRGTFASGNH	: 385
pig	: -----QHDAQNSGLFPVASIFKGLTSDNR	: 351
cattle	: -----QHDTQNSGLLIASIFKKELTSDNR	: 351

chicken	: PSSGQVSLEDQMLGIYPEALHEDDTRFLFGTGVSVLILSVLVILMFKK	: 574
human	: G-----DIGLLLGMIYFAVMLSILSLIGIFNR	: 378
mouse	: Q-----DIGLLSGMVFALIMLPFISLIGIFNR	: 412
pig	: QQ-----DIGLLGMVFFAVLLSILSLIGIFNR	: 379
cattle	: K-----DIGLLLGMIYFAVMLSILSLIGIFNR	: 378

chicken	: SARKRIKAAVALVLPKWLLDEPHMNSKVIKSEOEKSEFTNSIFSEPFL	: 624
human	: SERTGIKRRILLIPKWLYEDIPNMKNSNVVKMLQENSELMNNNSSEQVL	: 428
mouse	: SERTGIKRRVLLMIPKWLYEDIPNMENSNVAKLQEKSVFENDNASEQAL	: 462
pig	: SERTGIKRRILLIPKWLYEDIPNMENSHVVKMLQEKSEFMNNNSSKQVL	: 429
cattle	: SERTGIKRRILLIPKWLYEDIPNMENSKVVKILQERNEFMNNNSSEQVL	: 428

*

chicken	: DNSDPTITETIKETISGDKRYKNVDIKKEPSNVVPEINVEHPOSSAPPHNTAM	: 674
human	: Y-VDPMITEIKETIPIE-HKPTDYKKE-NTGPTETRDYPONSLFDNTTVV	: 475
mouse	: Y-VDPVLTEISETISPLE-HKPTDYKEERLTGLLETRDCPLGMLSTSSSVV	: 510
pig	: Y-VDPVITEI-EIIFLPE-EKPMGMKKEKNTGSLTFNSLOTSLSLNTTVV	: 476
cattle	: Y-VDPVITEI-EIIFLPE-EKPMGMKKENNTGCLERKESLEKSLTLDATTVV	: 475

*

chicken	: ED--VSLYKPOISDANTLYVAANMGLIQYIPAEPEPN-----IFFR	: 716
human	: YIPDLNTGYKPOISNLFPEGSHLSNNNEITSLTKPEVDSLDGNNPRLO	: 525
mouse	: YIPDLNTGYKPOVSNVPPGGLFINRDERDPTSLTETDDHF-----ARLK	: 555
pig	: YIPDLNTGYKPOISNLFITGSHLSNNDETASSILEPPADALNLGKNARFK	: 526
cattle	: YIPDLNTGYKPOISSFLPGSHLSNDETASSILEPPADSLNLGNNARFK	: 525

* *

chicken	: IYSSPESCLWDAEGAGAQVCLVDKINLVLNNDRSQGONHAFSSAQEEQ-NA	: 765
human	: KHEN-FAFSVSSVNSLSNTLFLGELSLILNOGECSSPDIONSVEEET-TM	: 573
mouse	: TYEN-EQFSASSMALLNKTLILDELCLVLNOGECFNSLDIKNSROEET-SI	: 603
pig	: KHPH-FAFSVSSMTSLSLNTLFLDELSLILNOGECSPDIONSIEGETTVQ	: 575
cattle	: KYPD-FAFSVSSVNSLSNTLFLDELNLILNOGECSPDMDONSTEGET-AM	: 573

*

chicken	: LLENQWEKTIYSEGVOEOTLVDELVSCLRAMNGGSVDITQTCFPOSTIGRL	: 815
human	: LLE----NDSPSETIPEQTLLPDEFVSCLGIVNEELPSINTYFPONILES	: 619
mouse	: VLQ----SDSPSETIQAOTLLPDEFVSCLAIGNEDLPSINSYFPONVLES	: 649
pig	: LLE----NASPNETIPEQTLLPDEFVSCLGIVNTELPINSYFPONILES	: 621
cattle	: LLE----DALLNETIPEQTLLPDEFVSCLGIVNTELPINSYFPONILES	: 619

* *

chicken	: F-----	: 816
human	: HENRISLLEK	: 629
mouse	: HESRISLFOK	: 659
pig	: HENRISLLEK	: 631
cattle	: HENRISLLEK	: 629

Figure 5.3. Comparison of the chIL-23R aa sequence with human, mouse, pig and cattle IL-23R aa sequences. Shaded areas represent conservation of aa similarity, the darker the shading the more conserved the residue is across the species. The vertical black and red arrows indicate the predicted signal sequence cleavage site of mammalian and chicken IL-23R respectively. Red boxes indicate mammalian N-linked glycosylation sites. Blue boxes indicate chicken N-linked glycosylation sites. Conserved cysteine residues are identified by a black asterisk. The horizontal black line indicates the WSXWS-like motif. The green box highlights the transmembrane domain. In the cytoplasmic domain, red asterisks indicate conserved mammalian tyrosine residues and the red circles highlight chicken tyrosine residues.

5.3.3 Alignment of the *chIL-23R* CDS with predicted *chIL-23R* nt sequences

As the annotated *chIL-23R* nt sequence (Ensembl) appeared to be incorrectly annotated, and the confirmed *chIL-23R* nt sequence was based on the GenScan prediction (GENSCAN00000023581), the three nt sequences were aligned for comparison. This confirmed that the annotated *chIL-23R* (ENSGALT00000018285) in Ensembl is incorrect and that the *chIL-23R* nt sequence is shorter than the GenScan prediction (GENSCAN00000023581), suggesting it is over predicted (Figure 5.4).

Chapter 5 Identifying the IL-23R in the chicken

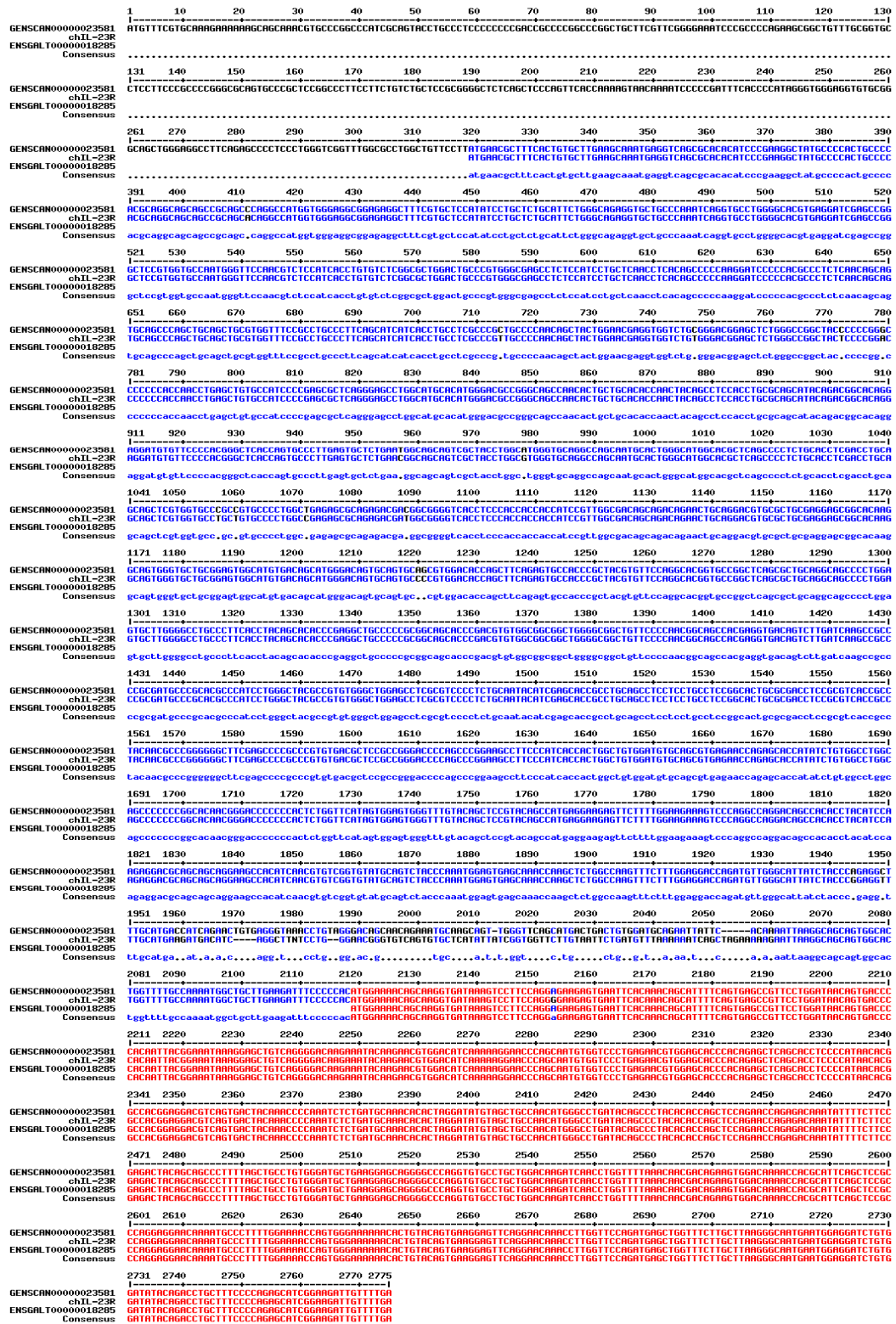


Figure 5.4. Nucleotide sequence alignment of the predicted chIL-23R (GENSCAN00000023581), the confirmed chIL-23R and the incorrectly annotated chIL-23R (ENSGAL00000018295). Identical nt sequence is red, partial nt alignment is blue and no alignment is black.

5.3.4 Gene organisation of the *chIL-23R*

Nucleotide BLAST analysis against the chicken genome (Galgal 4.0, Ensembl) shows that the *chIL-23R* is encoded by 15 exons (Figure 5.6) spanning 11 kb of chicken genomic DNA and is located on chromosome 8, 1138 nt upstream of the current annotated *chIL-23R* (ENSGALT00000018285) (Figure 5.5). The location of the gene for IL-12R β 2 (ENSGALT00000018300) in the chicken is also included, as it is found downstream on the same chromosome as in mammals, demonstrating conserved synteny in the chicken. In the genomic sequence of the *chIL-23R*, the intron splicing consensus (GT/AG) is conserved at the 5' and 3' ends of the introns (Appendix 6).

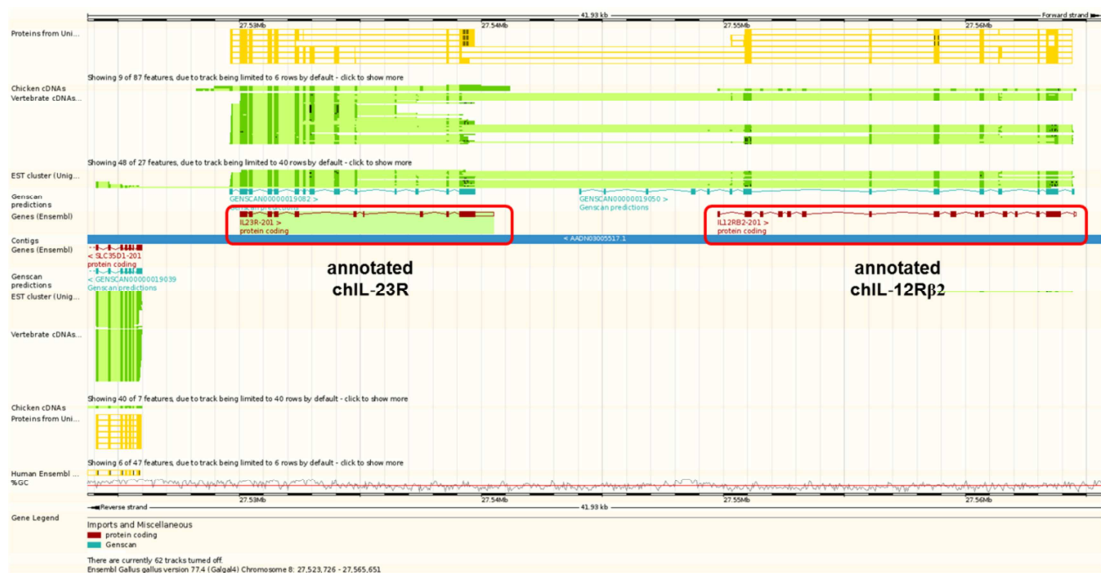


Figure 5.5. Ensembl (Galgal 4.0) showing the genetic location of the incorrectly annotated *chIL-23R* (ENSGALT00000018285) on chromosome 8 of the chicken genome. Red boxes highlight the incorrectly annotated *chIL-23R* (ENSGALT00000018285), and the annotated gene for *chIL-12R β 2* (ENSGALT00000018300), located downstream on the same chromosome.

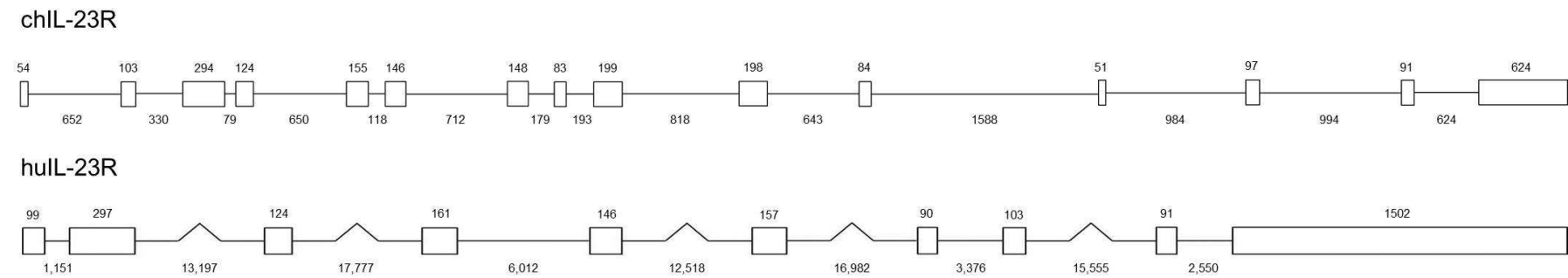


Figure 5.6. Exon-intron organisation of the chicken IL-23R (chIL-23R) compared to the human IL-23R (huIL-23R). Exons are represented by open boxes and introns are represented by black lines. The number of nucleotides in each exon is shown above the gene structure and the number of nucleotides in each intron is shown below the gene structure. The chIL-23R is encoded by 15 exons and the huIL-23R is encoded by 10 exons.

5.3.5 Expression of *chIL-23R* mRNA in tissues

Having confirmed the *chIL-23R* nt sequence, it was now possible to measure mRNA levels of the *chIL-23R* in various tissues. The *chIL-23R* is constitutively expressed in all tissues at moderate levels (5-15 corrected 40-Ct) (Figure 5.7). The highest mRNA levels in non-lymphoid tissues were in the heart, lung and gut and in lymphoid tissues were in the caecal tonsil.

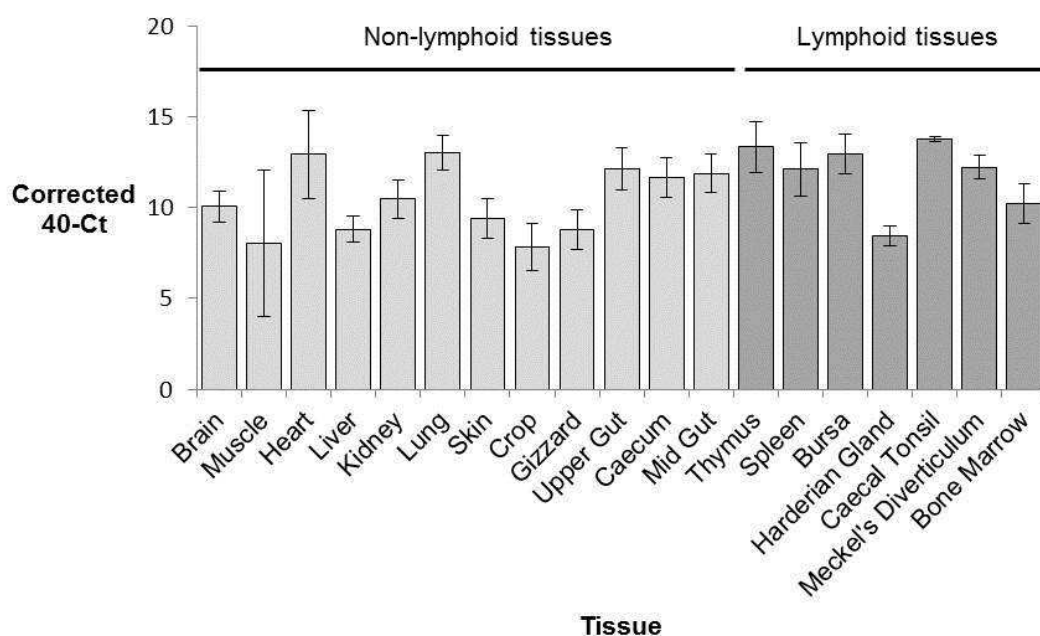


Figure 5.7. Expression patterns of *chIL-23R* mRNA in tissues as measured by RT-qPCR. Data are expressed as corrected 40-Ct values \pm SEM. Assays were carried out in triplicate and each value is the mean of 3 birds.

5.3.6 Expression of *chIL-23R* mRNA levels in stimulated splenocytes, thymocytes and bursal cells across a time course

Chicken IL-23R mRNA levels were measured in stimulated lymphoid cells and compared to levels in unstimulated controls. Splenocytes were stimulated with ConA, thymocytes with PHA and bursal cells with PMA and ionomycin over a time course of 2-24 h. Chicken IL-23R mRNA levels in stimulated splenocytes were significantly up-regulated compared to unstimulated controls at 12 h post-stimulation ($p = 0.014$) (Figure 5.8 (A)). However, there was no significant relationship in *chIL-23R* mRNA expression in stimulated splenocytes over time. In stimulated thymocytes, *chIL-23R* mRNA levels were significantly up-regulated compared to unstimulated controls at 18-24 h post-stimulation ($p < 0.021$) (Figure 5.8 (B)). Further, there was a significant positive linear relationship ($p = 0.040$) in *chIL-23R* mRNA fold change over time, with *chIL-23R* mRNA levels increasing from 2-24 h post-stimulation. In stimulated bursal cells, *chIL-23R* mRNA levels were significantly up-regulated at 4-12 ($p < 0.031$) and 24 h ($p = 0.002$) post-stimulation (Figure 5.8 (C)) but there was no significant relationship in *chIL-23R* mRNA expression over time.

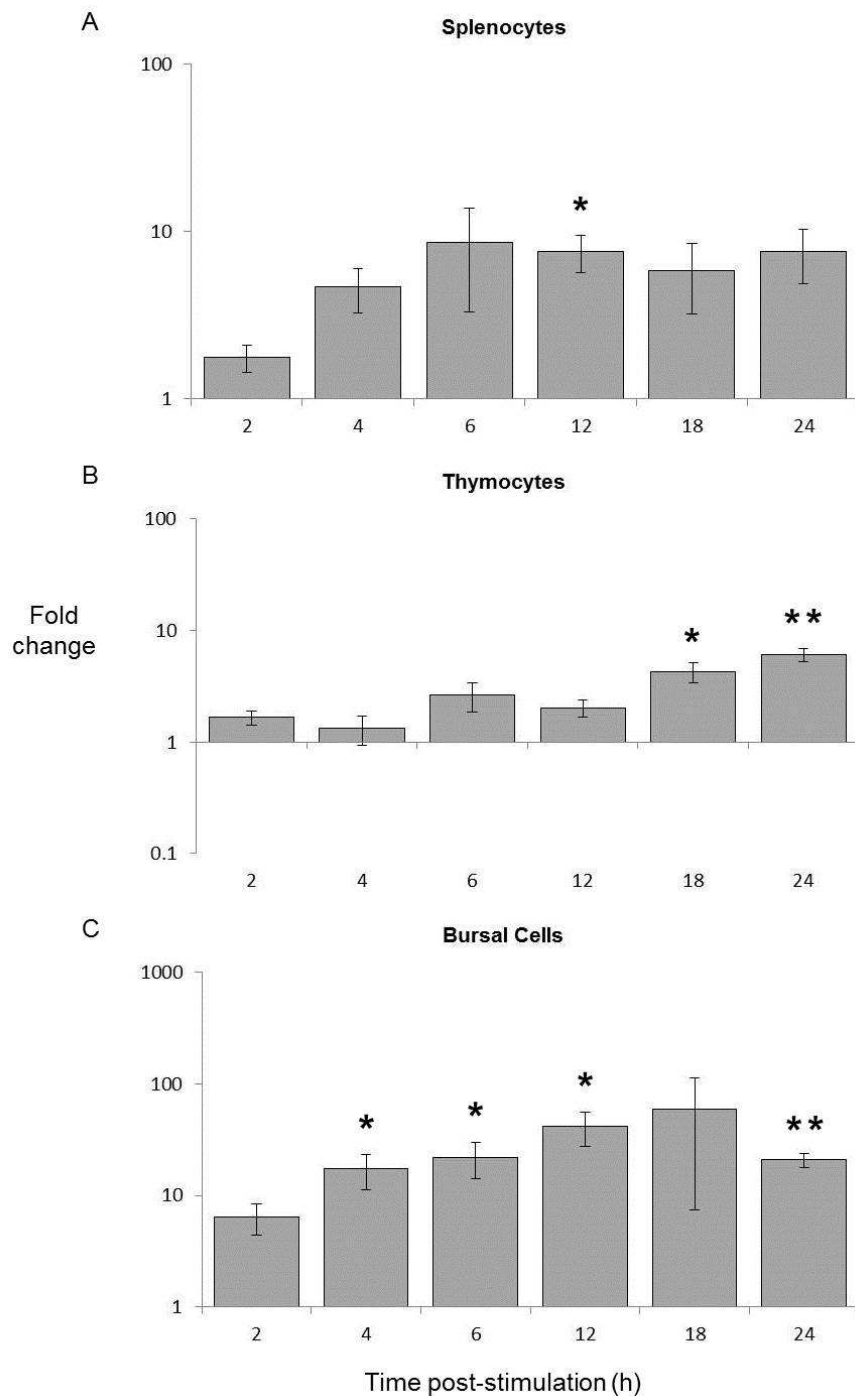


Figure 5.8. Expression patterns of chIL-23R mRNA in stimulated (A) splenocytes, (B) thymocytes and (C) bursal cells. Data are expressed as fold change compared to levels in age-matched unstimulated controls \pm SEM. A linear mixed effect model was used to determine if there was a relationship between mRNA expression levels over time. A standard one-sample t test was performed to determine if cytokine levels were significantly up or down-regulated relative to a fold change of one as indicated by a single asterisk ($p < 0.05$) or double asterisk ($p < 0.01$). Chicken splenocytes, thymocytes and bursal cells were stimulated with ConA, PHA and PMA and ionomycin respectively for between 2-24 h. Assays were carried out in triplicate and each value is the mean of 3 birds.

5.4 Discussion

In humans and mice Th17 cells require IL-23 for stabilisation of the Th17 phenotype (McGeachy *et al.*, 2009). The expression of IL-23R is driven by STAT3 activation by IL-1 β and IL-6 (Durant *et al.*, 2010; Ivanov *et al.*, 2006). In addition, IL-23 also markedly increases expression of the IL-23R, in an autocrine manner (Ghoreschi *et al.*, 2010). The IL-23R in mice and humans was identified shortly after IL-23 was discovered, by expression cloning e.g. screening a cDNA library with a bioassay (Parham *et al.*, 2002). IL-23R pairs with IL-12R β 1 to confer responsiveness to IL-23 in cells expressing both receptor subunits. As Th17 cells universally express the IL-23R (McGeachy *et al.*, 2009), it is used as a cell-surface marker for identification of Th17 cells. The IL-23R is currently incorrectly annotated in the chicken genome. Therefore, this Chapter describes the identification and initial characterisation of the chIL-23R, in order to facilitate the identification of Th17 cells in the chicken in the future.

The initial aim of this Chapter was to identify the correct chicken IL-23R gene. As previously discussed (Chapter 3), classical molecular approaches to characterise avian cytokines and their receptors are not usually successful due to low sequence homology with mammalian orthologues. The CDS for chIL-23R was identified using *in silico* and *in vitro* methods. Due to the GC-rich areas of the chIL-23R nt sequence, the chIL-23R CDS was difficult to amplify by PCR. Multiple primer sets of varying lengths and GC content, covering different parts of the predicted sequence were used to amplify the chIL-23R. Different cDNA templates, using all possible combinations of forward and reverse primers, as well as different annealing temperatures and extension times for the various primer combinations were also used to confirm the

chIL-23R CDS. Subsequently, using increased amounts of RNA in cDNA synthesis and a high MgCl₂ concentration, the full-length sequence of the chIL-23R was confirmed. This also confirmed the predicted nt sequence for chIL-23R (GENSCAN00000023581) to be over predicted and the annotated nt sequence for chIL-23R to be under predicted (ENSGALT00000018285). However, the genome location of the chIL-23R is correct (chromosome 8) and demonstrates conserved synteny with mammals, the chIL-23R being located upstream of the chIL-12R β 2 on the same chromosome. The gene organisation of the chIL-23R is different to mammalian IL-23Rs. The chIL-23R is encoded by 15 exons spanning 11 kb of chicken genomic DNA. This was confirmed by comparison of the chIL-23R nt sequence and the chicken genome sequence identifying the intron splicing consensus (GT/AG) at the 5' and 3' ends of the introns (Appendix 6). The human IL-23R was originally thought to be encoded by 10 exons spanning 92 kb of human genomic DNA (Parham *et al.*, 2002). However, it is now evident that the human IL-23R mRNA is comprised of 11 exons (Kan *et al.*, 2008). The cattle and pig IL-23Rs are encoded by 10 exons and 11 exons respectively. Multiple splice forms of the human IL-23R have been identified (Kan *et al.*, 2008; Zhang *et al.*, 2006) so it is possible that the identified chIL-23R is not a functional protein, and a shorter splice variant is expressed as the functional isoform of the chIL-23R. Further investigation by using restriction enzyme digestion and sequence analysis is required to establish if there are multiple splice variants of the chIL-23R as in humans.

Following identification of the chIL-23R gene, the aa sequence was compared to its mammalian counterparts. Chicken IL-23R is longer in aa sequence length compared to mammalian IL-23R proteins. The intracellular and transmembrane domains of

mammalian and chIL-23R are of similar aa length but the aa sequence of the chIL-23R extracellular domain is longer than its mammalian counterparts. A functional reason for this is unclear at the moment. The crystal structure of human IL-23 is available (Beyer *et al.*, 2008; Lupardus and Garcia, 2008) but the crystal structure of mammalian IL-23R has not been investigated. Elucidating the crystal structure of the chIL-23R and comparing to mammals would be interesting and may shed light on potential functional differences in the IL-23R between birds and mammals, as well as a reason for the longer aa sequence of the chIL-23R.

Despite the chIL-23R aa sequence being longer, there is an expected level of aa identity (27.1%) between the chIL-23R and mammalian IL-23R aa sequences. The predicted signal sequence cleavage site in the chIL-23R is similar to the known signal sequence cleavage site in the mammalian IL-23R. Eleven of the 19 cysteine residues in the chIL-23R are conserved between chicken and mammals, and there is one conserved potential N-linked glycosylation site. N-linked glycosylation is an important post-translational modification during protein expression and is important in protein folding and the stability of expressed proteins (Mitra *et al.*, 2006). It is also important for ligand binding and stability of cytokine receptors (Ding *et al.*, 1995). The transmembrane domain of the chIL-23R contains a sequence (WSAWG) similar to the cytokine receptor signature WSXWS-like motif, which is characteristic of the mammalian type I cytokine receptor superfamily (haemopoietin receptor family). In the intracellular domain of the chIL-23R, there are two tyrosine residues, which are conserved with mammals. The number of tyrosine residues (six) in the chIL-23R suggests a role in the signal transduction process of the receptor as in mammals. It is difficult to discern a proline-rich area in the transmembrane domain of the chIL-23R

that would be predicted to bind a Jak. To summarise, the IL-23R has been identified in the chicken genome but the possibility of additional isoforms of this receptor requires further investigation.

To investigate the potential role of IL-23 and the IL-23R in the chicken, expression of the chIL-23R was investigated in a broad range of tissues and stimulated cells. Chicken IL-23R mRNA was measured in all tissues. In humans, IL-23R mRNA expression is primarily restricted to the lymph nodes, the gut and the lungs (Shmueli *et al.*, 2003; Yanai *et al.*, 2005). In IL-23R-eGFP mice, gut-associated lymphoid tissue exhibited the highest number of IL-23R⁺ cells (Chognard *et al.*, 2014). The mRNA levels of the chIL-23R were also higher in the gut and the lung compared to other non-lymphoid tissues measured. IL-23 is produced early in response to pathogenic challenge and is essential for driving early local immune responses (McKenzie *et al.*, 2006). Therefore, it is not surprising to find increased expression of IL-23R in the mucosal tissues of the gut, allowing a rapid immune response in those tissues to expression of IL-23. The increased mRNA levels of the chIL-23R in the gut and lung suggests a similar role for the chIL-23R to mammals.

Messenger RNA levels of the chIL-23R were also measured in stimulated lymphoid tissues and compared to unstimulated controls, to further outline the potential role of the IL-23R during immune responses in the chicken. ConA stimulation of splenocytes increased mRNA expression of the chIL-23R but there was no relationship in chIL-23R expression over time. Expression of the IL-23R in stimulated cells has never been examined over a time course *in vitro* in any other species so it is difficult to draw comparisons. However, PHA-stimulated PBMCs showed enhanced expression of the IL-23R in the pig (Kokuho *et al.*, 2012). In

humans and mice, the IL-23R is expressed predominantly on T cells ($\gamma\delta$ T cells and Th17 cells) (Chognard *et al.*, 2014). Compared to mice and humans, chickens possess a higher number of $\gamma\delta$ T cells (Su *et al.*, 1999), which comprise approximately 20% of the total splenocyte population (Pieper *et al.*, 2008). ConA stimulation of splenocytes may be increasing mRNA expression of the chIL-23R in $\gamma\delta$ T cells. In mice and humans, IL-23R is quite lowly expressed on cells and there are a lack of Ab reagents to identify and track IL-23R-positive cells *in vivo* (Awasthi *et al.*, 2009). Development of a chIL-23R monoclonal Ab would potentially allow for investigation of the specific cell types which express the chIL-23R and a comparison of these cells to humans, therefore, confirming if chicken T cells also express the chIL-23R.

Expression of chIL-23R mRNA in stimulated thymocytes and bursal cells was increased compared to unstimulated controls. Chicken IL-23R mRNA expression increased over time in stimulated thymocytes but this relationship was not seen in stimulated bursal cells. As the IL-23R is expressed predominantly on mature T cells in mammals, it is unclear which cell types in the chicken thymus are expressing the chIL-23R following stimulation. The IL-23R is expressed by a small number of B cells in mice (Chognard *et al.*, 2014), which were identified using the IL-23R-eGFP mouse (Awasthi *et al.*, 2009). Also, IL-23 has been identified in the human tonsillar B cell response through expression of the IL-23R on plasma cells (Cocco *et al.*, 2011). These data suggest chicken B cells express the chIL-23R. However, the specific cell types which express the chIL-23R need to be identified in order to investigate the biological role of chIL-23 on B cells. These data suggest thymocytes and bursal cells are potentially capable of responding to IL-23-stimulation. Further

investigation of the specific cell types involved and whether these cells co-express chIL-12R β 1 is required.

In mammals, the IL-23R is made up of 2 subunits, IL-23R and IL-12R β 1, and co-expression of both subunits allows a cell to respond to IL-23. The mRNA expression of chIL-12R β 1 has not been investigated in the chicken. Further study is needed to investigate if cells which co-express the chIL-23R and chIL-12R β 1 respond to chIL-23 similarly to mammals. In mammals, IL-12R β 1, the sub-unit common to both IL-12 and IL-23R, is expressed in all sorted populations of PBMCs (Chognard *et al.*, 2014), whereas the expression of IL-23R and IL-12R β 2 is restricted and defines the cell types responding to either IL-23 or IL-12 signalling. The IL-23 and IL-12 receptors are not expressed on the same cell types in mammals and show a strong dichotomy in the cells which express the IL-12R or the IL-23R. Most notably, the receptors for IL-12 and IL-23 only appear to be co-expressed in $\gamma\delta$ T cells, of which the chicken has high circulating numbers. It would be interesting to investigate the specific cell types expressing the chIL-23R and the chIL-12R for comparison to mammals, as the cell populations that express either the IL-12R or the IL-23R are well-conserved between mouse and man (Chognard *et al.*, 2014).

Whilst expression of mRNA is informative as to which cells express IL-12R or IL-23R, it does not necessarily correspond to expression of functional protein. Therefore, in order to identify specific cells expressing the functional IL-23R, the development of a monoclonal antibody to the chIL-23R is needed. It was hoped that development of a chIL-23R monoclonal antibody would be possible during my PhD, unfortunately due to time constraints, it has not been and development of this useful reagent remains the remit of future work. A chIL-23R monoclonal Ab would

potentially allow identification of Th17 cells in the chicken. Positively identifying Th17 cells in the chicken would confirm that chickens mount Th17 immune responses in a manner similar to mammals. Th17 cells in mammals can also be identified by expression of IL-17A, ROR γ t and CCR6 (Acosta-Rodriguez *et al.*, 2007b; Harrington *et al.*, 2005; Ivanov *et al.*, 2006). IL-17A and CCR6 are expressed in the chicken (Min and Lillehoj, 2002; Munoz *et al.*, 2009) but ROR γ t is not currently annotated in the chicken genome. Identifying ROR γ t in the chicken and development of monoclonal Ab for chROR γ t and chCCR6, as well as using the chIL-17A Abs (Yoo *et al.*, 2008), would allow for thorough investigation and identification of the chicken cell types co-expressing these molecules and a comparison to mammals.

To summarise, the IL-23R has been identified and cloned in the chicken. The chIL-23R was expressed in a wide range of tissues and was up-regulated in stimulated splenocytes, thymocytes and bursal cells. Confirmation that the chIL-23R is in the chicken genome, and is expressed in tissues and stimulated cells in the bird, is highly suggestive of Th17 immune responses in the chicken. The identification of the chIL-23R is the beginning to developing useful tools to further identify Th17 cells in the chicken and confirming that chickens mount Th17 immune responses in similar manner to mammals.

Chapter 6 Differentiating Th17 cells in the chicken

6.1 Introduction

Th cells are distinguished into different lineages based on their pattern of cytokine secretion, transcription factor expression, and functions, which are responsible for different immune responses to specific pathogens as well as immunopathology. The original Th1/Th2 paradigm as proposed by Mosmann and Coffman (1989) defined Th1 cells as producing IFN- γ , and protecting against intracellular infections, and Th2 cells as producing IL-4, IL-5 and IL-13, and protecting against extracellular infections. By this definition, the Th1/Th2 paradigm applies in the chicken (Degen *et al.*, 2005; Powell *et al.*, 2009). However, the repertoire of mammalian CD4⁺ T cell subsets has now expanded beyond the original paradigm to include regulatory subsets and other effector subsets (reviewed by Kara *et al.*, 2014). The understanding of CD4⁺ cellular subsets and the responses in which they are involved in the chicken naturally lags behind that in biomedical model species (Kaiser, 2010), expanding beyond the Th1/Th2 paradigm to include the recent identification of Tregs in the chicken (Shanmugasundaram and Selvaraj, 2010; 2011).

The concept of an IL-17/IL-23 pro-inflammatory axis in mammals was first suggested by Aggarwal *et al.* (2003) but it was Harrington *et al.* (2005) who described the Th17 cell. The Stockinger group were first to differentiate Th17 cells *in vitro* from naïve CD4⁺ T cells using TGF- β and IL-6 (Veldhoen *et al.*, 2006). Th17 cell differentiation can be split into 3 stages: the differentiation stage mediated by TGF- β and IL-6, the self-amplification stage by IL-21, and the stabilisation stage by IL-23 (Luckheeram *et al.*, 2012). However, whilst the addition of TGF- β and IL-6

into naïve CD4⁺ T cell cultures does indeed drive Th17 cell differentiation, the *in vivo* requirements of Th17 cell differentiation are far more complex than these *in vitro* conditions. A detailed description of mammalian Th17 differentiation is discussed in Chapter 1. *In vitro* studies in mammals using Th17 cells differentiated *ex vivo* from naïve CD4⁺ T cells commonly use CD3 and CD28 antibodies to mimic the two-signal activation of naïve T cells required for T cell expansion (Trickett and Kwan, 2003). Specific stimulation for a Th17 phenotype is provided by the recombinant cytokines TGF- β and IL-6 with or without the addition of IL-1 β , IL-23 and IL-2 (Lee *et al.*, 2012; Mangan *et al.*, 2006; Veldhoen *et al.*, 2006; Wilson *et al.*, 2007). Further, antibodies against IL-12, IFN- γ , and IL-4 can also be used to prevent cells differentiating towards a Th1 or Th2 phenotype (Ivanov *et al.*, 2006; Zhou *et al.*, 2008). Th17 cells are identified by the expression of the T cell marker CD4 as well as IL-17A and/or the IL-23R (Harrington *et al.*, 2005; Veldhoen *et al.*, 2006; Wilson *et al.*, 2007).

Due to a greater repertoire of reagents available in mammalian immunology, differentiating Th cells *in vitro* and identifying Th cells *in vivo* in mice and man is now commonplace. However, *in vivo* identification of specific cell types is still a challenge in the chicken and *in vitro* differentiation of Th cells has not been possible to-date. In order to answer the question are there Th17 immune responses in the chicken, this Thesis has identified the key missing cytokine, IL-23 and demonstrated a similar biological activity to mammals (Chapter 3), measured mRNA expression the Th17 effector cytokines in tissues, stimulated cells and during infection in the chicken (Chapter 4) and identified a phenotypic marker for Th17 cells, the IL-23R (Chapter 5). The aim of this Chapter is to differentiate chicken Th17 cells *in vitro*

from naïve CD4⁺ T cells using recombinant cytokines, in a similar method to mammals, and measure mRNA expression of the Th17 effector cytokines and the IL-23R to confirm a Th17 phenotype.

6.2 Methods

General methods are outlined in Chapter 2. Additional methods and alterations are detailed here.

6.2.1 *Optimisation of splenocyte co-stimulation assay*

Anti-chicken CD3 (AV36 and Southern Biotech clone CT-3) and CD28 antibodies (AV7) were immobilised on a flat-bottomed 96-well tissue culture plate. CD3 antibodies were diluted to 1 µg/ml in sterile PBS. CD28 antibody was then diluted 1/1000, 1/500 or 1/250 in 1 ml of 1 µg/ml CD3 antibody solution. The antibody solution, or sterile PBS as a control (50 µl per well) was added to each well of the 96-well plate and incubated for 3 h at 37°C. The plate was then vigorously washed three times with sterile PBS to remove any unbound antibodies. Chicken splenocytes were isolated as described in section 2.7.1 and adjusted to a density of 5 x 10⁶ cells/ml. The cell suspension (100 µl/well) was added to the antibody-coated plate with or without the addition of ConA (0.5 µg/ml), with complete medium alone (Appendix 1) as a control. The cells were cultured at 41°C, 5% CO₂ for 72 h. After 72 h of culture, 20 µl of CellTiter 96 Aqueous One Solution (Promega) was added to each well and the plate incubated for a further 4 h. After 4 h of culture, the absorbance of the plate was measured at 490 nm on 96-well plate reader.

6.2.2 Optimisation of MACS sorted CD4⁺ T cell stimulation

Further to the stimulation of CD4⁺ T cells outlined in section 2.11.3, CD4⁺ T cells were stimulated with ConA (1 and 2 µg/ml) for 4, 24 and 72 h.

6.3 Results

6.3.1 Optimisation of anti-CD3 (α CD3) and anti-CD28 (α CD28) stimulation

To drive CD4⁺ T cells towards a Th17 phenotype, CD3 and CD28 antibodies were used, in conjunction with Th17 driving cytokines, to stimulate the cells. The optimal concentrations of CD3 and CD28 antibodies for maximal splenocyte proliferation were 1 µg/ml of CD3 antibody (AV36) and a 1/250 dilution of CD28 antibody (AV7) (Figure 6.1).

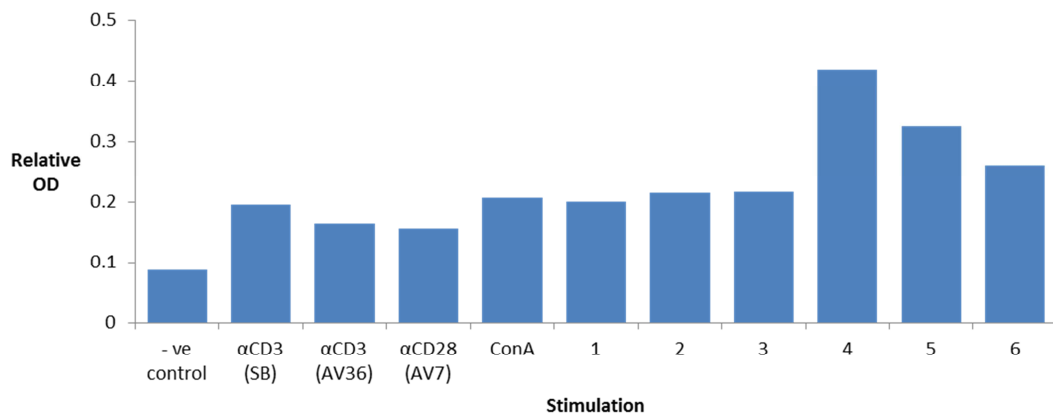


Figure 6.1. Measurement of splenocyte proliferation after α CD3 and α CD28 co-stimulation. Optical density (OD) was measured at 490 nm and corrected for medium alone (Relative). Splenocytes were stimulated for 72 h with a negative control (medium), α CD3 (1 µg/ml) from Southern Biotech (SB) or the AV36 clone (AV36), α CD28 (1/500 dilution) the AV7 clone (AV7), ConA (0.5 µg/ml) or a combination of α CD3 and α CD28 (1-6). 1) α CD3 (SB) and α CD28 (1/250). 2) α CD3 (SB) and α CD28 (1/500). 3) α CD3 (SB) and α CD28 (1/1000). 4) α CD3 (AV36) and α CD28 (1/250). 5) α CD3 (AV36) and α CD28 (1/500). 6) α CD3 (AV36) and α CD28 (1/1000). Highest splenocyte proliferation was measured in 4) α CD3 (AV36) and α CD28 (1/250). The data are representative of 1 bird.

6.3.2 FACS

Chicken CD4⁺ T cells were negatively sorted from splenocytes by fluorescence-activated cell sorting (FACS) (kindly carried out by Bob Fleming). The gating strategy employed is shown in Figure 6.2. Following removal of cells that stained positive for CD8 α (CD8⁺ T cells), TCR1 ($\gamma\delta$ T cells), KUL01 (macrophages) and Bu-1 (B cells), approximately 7% of the total cells were chicken CD4⁺ T cells. Following FACS analysis of the sorted chicken CD4⁺ T cells, the cells were > 80% pure. A total of 4.8×10^6 cells were collected from 9.6×10^7 cells.

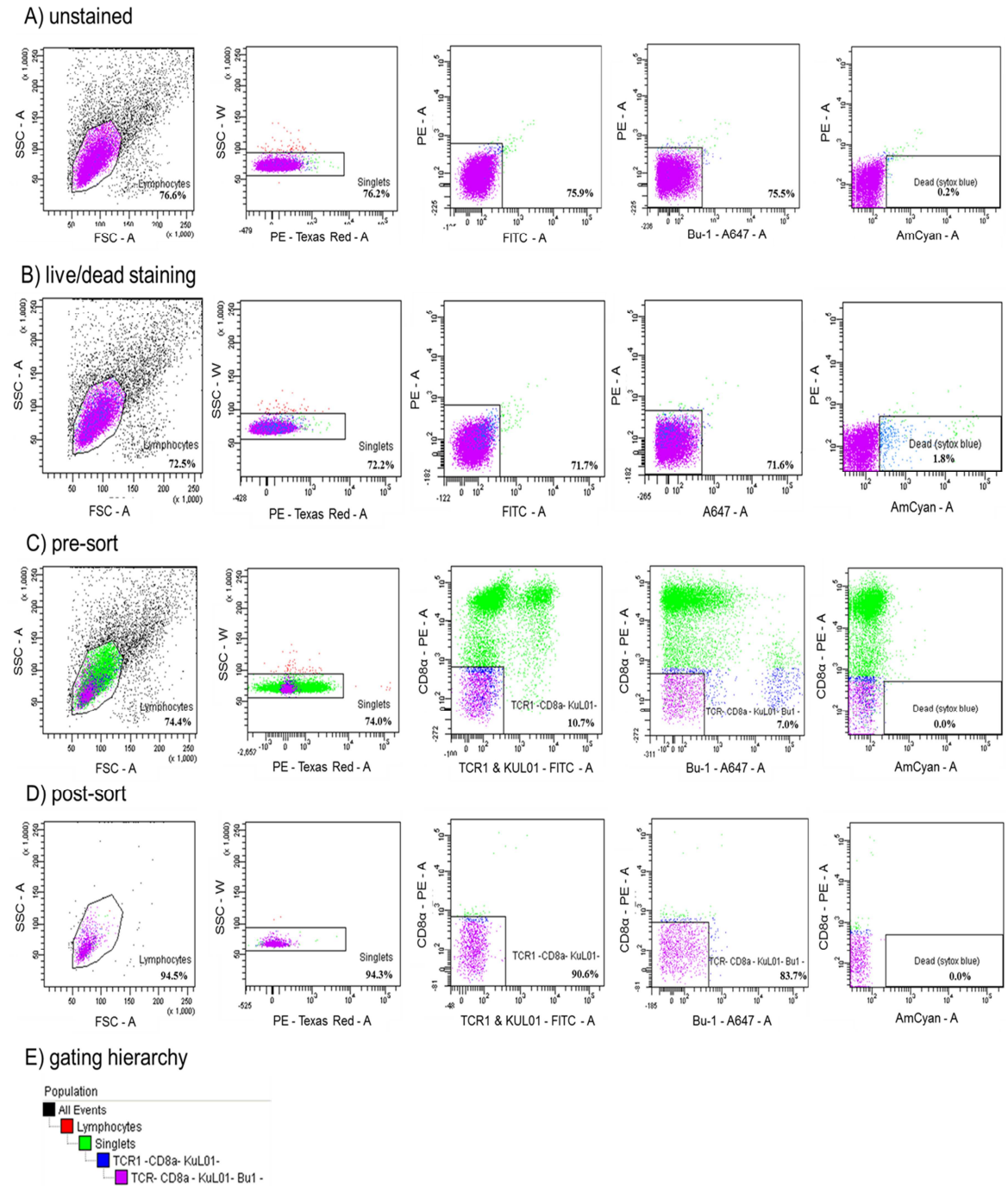


Figure 6.2. Negative FACS of chicken CD4⁺ T cells. FACS dot plots for A) unstained splenocytes, B) live/dead staining C) pre-sort and D) post-sort chicken CD4⁺ T cells and E) the gating hierarchy applied. Analysis of the side scatter (SSC) versus the forward scatter (FSC) plot identified lymphocytes. Single cells were identified by the SSC plot. Cells negative for CD8α, TCR1, KUL01 and Bu-1 Ab staining were subsequently selected for. Sytox blue staining of a sample of cells selected for live cells. Percentages (%) of cells are of the total cell population. The flow cytometric results here represent one experiment of 3 repeats.

Following pooling and centrifugation of all negatively sorted cells, no cell pellet was visible nor were any live cells visible following Trypan blue staining. The cell sort was repeated twice more using an identical gating strategy and all negative cells were collected. Using Trypan blue staining and a haemocytometer, the cells were counted (4.6×10^6 and 5.5×10^6 cells respectively).

Following FACS sorting, chicken $CD4^+$ T cells were driven towards a Th17 phenotype by TCR activation and cytokine stimulation. To confirm the chicken $CD4^+$ T cells had differentiated into a Th17 phenotype, mRNA levels of the reference gene 28S, and the cytokines chIL-17F and chIL-2 were measured from the control stimulated $CD4^+$ T cells. Messenger RNA expression of 28S was detected from two experiments but chIL-17F and chIL-2 mRNA expression was not measured (Figure 6.3), indicating the cells did not proliferate and were not stimulated.

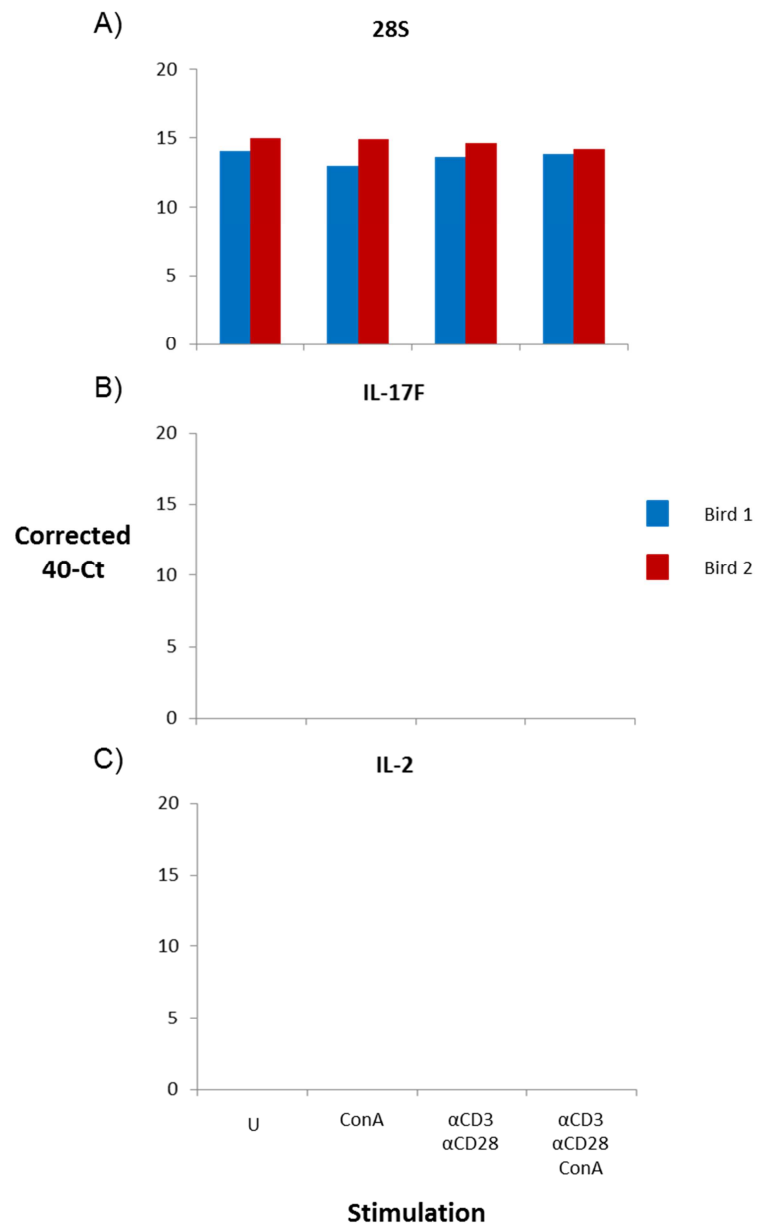


Figure 6.3. Expression of A) 28S, B) chIL-17F and C) chIL-2 mRNA in stimulated CD4⁺ T cells. Data are expressed as corrected 40-Ct. Chicken CD4⁺ T cells were stimulated with medium alone (U), ConA alone (ConA), CD3 and CD28 Ab together (αCD3 αCD28) or CD3 and CD28 Ab and ConA together (αCD3 αCD28 ConA) for 3 days. These data are representative of 2 birds. Technical replicates were carried out in triplicate. No chIL-17F or chIL-2 mRNA expression was detected.

Following 3 unsuccessful attempts to negatively sort chicken CD4⁺ T cells and drive them towards a Th17 phenotype, chicken CD4⁺ T cells were positively sorted by FACS (kindly carried out by Bob Fleming). The gating strategy used is indicated in Figure 6.4. Chicken T cells that stain positively for CD4 were selected for and approximately 14% of the total cell population were CD4⁺. When the positively sorted chicken CD4⁺ T cells were analysed by FACS, they were approximately 38% pure.

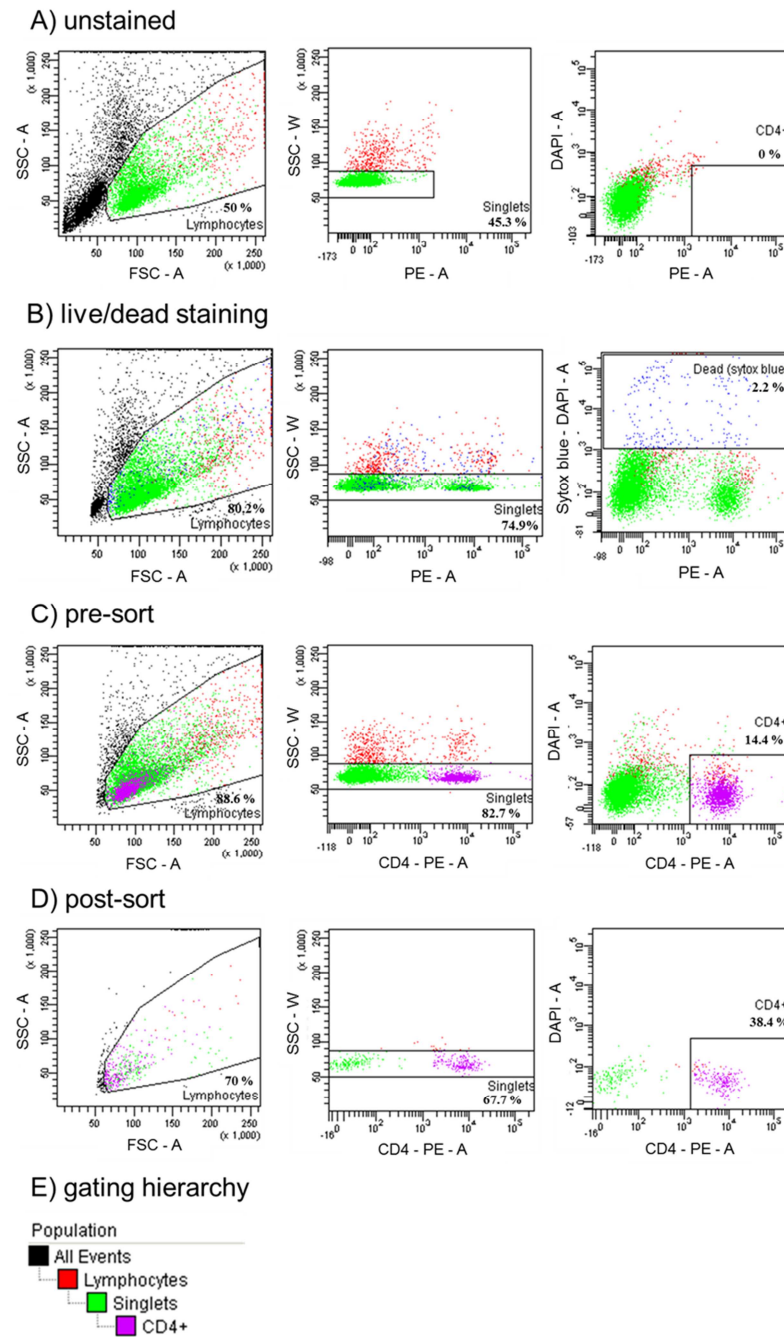


Figure 6.4. Positive FACS of chicken CD4⁺ T cells. FACS dot plots for A) unstained splenocytes, B) live/dead staining C) pre-sort and D) post-sort chicken CD4⁺ T cells and E) the gating hierarchy applied. Analysis of the side scatter (SSC) versus the forward scatter (FSC) plot identified lymphocytes. Single cells were identified by the SSC versus PE plot. Live cells negative for Sytox blue staining and positive for CD4 Ab staining were subsequently selected for. Percentages (%) of cells are of the total cell population. The flow cytometric results here represent one experiment.

Following pooling and centrifugation of all positively sorted $CD4^+$ cells, no cell pellet was visible nor were any live cells visible following Trypan blue staining. A sample of the collected cells was re-examined by FACS and the total percentage of chicken $CD4^+$ T cells was reduced 7-fold (38.4% compared to 4.9% of the total cells) compared to immediately post-sort (Figure 6.5), indicating a substantial loss of cells.

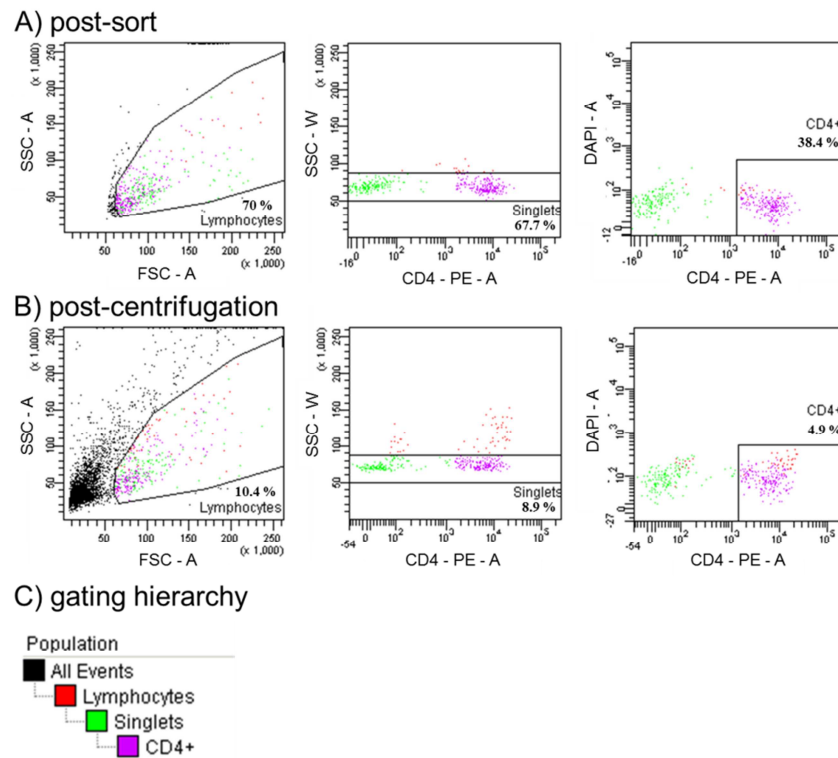


Figure 6.5. Comparison of chicken $CD4^+$ T cells A) immediately post-sort and B) following centrifugation with C) the gating hierarchy used. Percentages (%) of cells are of the total cell population. FACS dot plots show a 7-fold reduction in all cells after centrifugation to collect the sorted chicken $CD4^+$ T cells. The flow cytometric results here represent one experiment.

6.3.3 MACS

Chicken CD4⁺ T cells were positively sorted by magnetic-activated cell sorting (MACS). To assess the purity of the MACS sorted cells, they were analysed by FACS (kindly carried out by Lonneke Vervelde) (Figure 6.6). Chicken T cells that stain positively for CD4 were selected for and > 83% of the live MACS-sorted cell population was CD4⁺. These data are representative of 2 repeats.

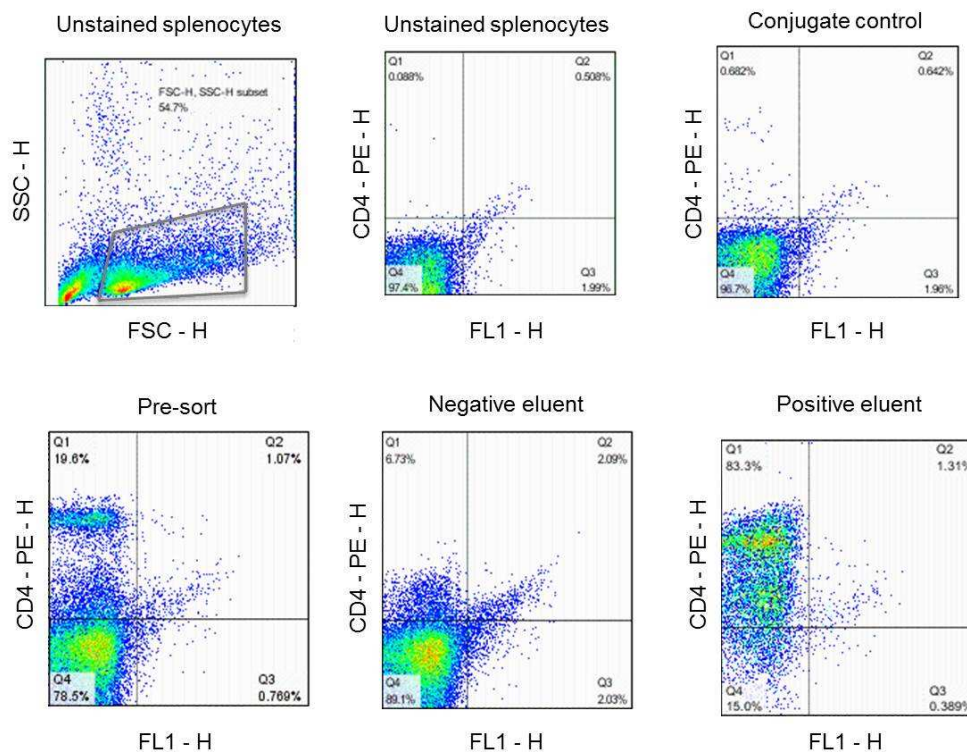


Figure 6.6. FACS analysis of MACS of chicken CD4⁺ cells. The SSC versus FSC plot was used to select for the live cell population in the unstained splenocytes (54.7%). The unstained cell population was confirmed CD4⁺ and the Ab (goat anti-mouse IgG1) used as a conjugate control was also negative. Negative CD4 cells post-sort contained 6.7% CD4⁺ cells. Chicken CD4⁺ cells were 83% of the live cell population.

Following MACS sorting, using Trypan blue staining, the number of sorted cells was counted (3.5×10^7 cells) and subsequently chicken CD4⁺ T cells were driven towards a Th17 phenotype by TCR activation and cytokine stimulation. To identify Th17 cells, mRNA levels of the reference gene 28S, and the cytokines chIL-17F and chIL-2 were measured from the control stimulated CD4⁺ T cells after 5 d of stimulation (Figure 6.7). Figure 6.7 (A) shows low levels of 28S mRNA was measured but mRNA expression of chIL-17F and chIL-2 was not detected, indicating the cells did not proliferate and were not stimulated.

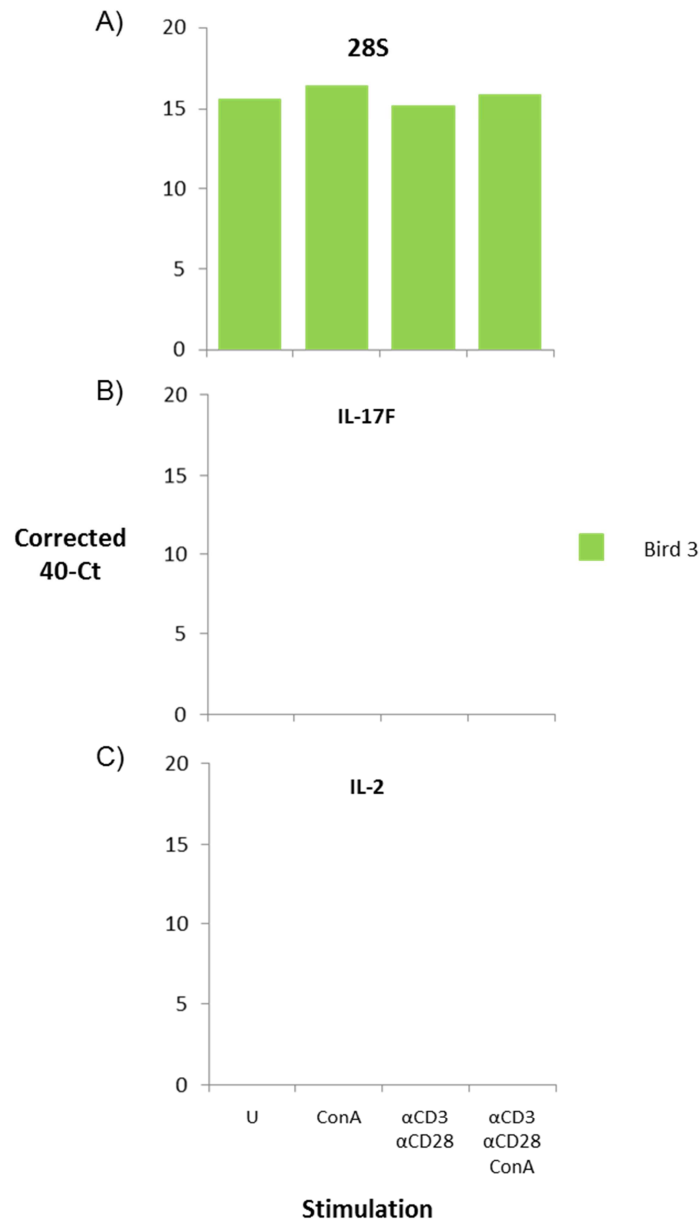


Figure 6.7. Expression of A) 28S, B) chIL-17F and C) chIL-2 mRNA in stimulated CD4⁺ T cells. Data are expressed as corrected 40-Ct. Chicken CD4⁺ T cells were stimulated with medium alone (U), ConA alone (ConA), CD3 and CD28 Ab together (αCD3 αCD28) or CD3 and CD28 Ab and ConA together (αCD3 αCD28 ConA) for 5 days. These data are representative of 1 bird. Technical replicates were carried out in triplicate. No chIL-17F or chIL-2 mRNA expression was detected.

Additionally, the MACS sorted CD4⁺ T cells were also stimulated with a higher concentration of ConA (2 µg/ml) for 4 h and the mRNA levels of 28S, chIL-17F and chIL-2 also measured (Figure 6.8). Low mRNA levels of 28S were expressed (Figure 6.8 (A)) but chIL-17F and chIL-2 mRNA expression was not detected. Data shown in Figure 6.8 are from 4 h of stimulation but are representative of 24 and 72 h. The chicken CD4⁺ T cells did not proliferate and were not stimulated.

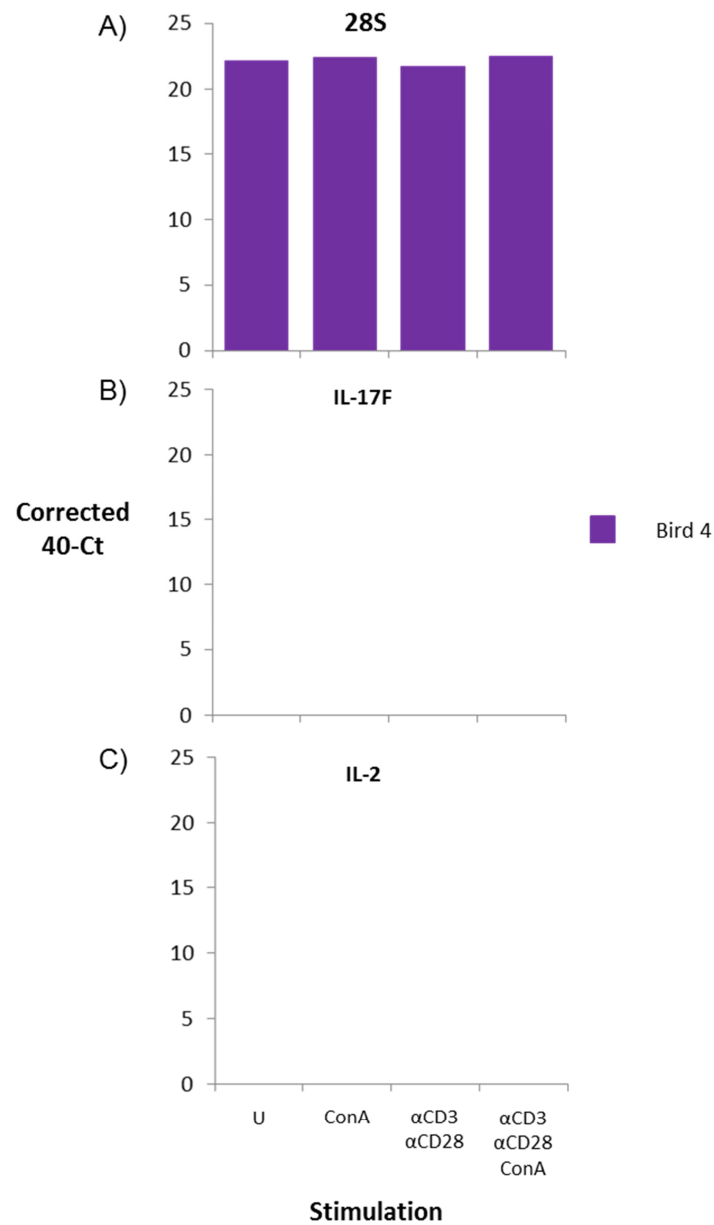


Figure 6.8. Expression of A) 28S, B) chIL-17F and C) chIL-2 mRNA in stimulated CD4⁺ T cells. Data are expressed as corrected 40-Ct. Chicken CD4⁺ T cells were stimulated with medium alone (U), ConA alone (ConA), CD3 and CD28 Ab together (αCD3 αCD28) or CD3 and CD28 Ab and ConA together (αCD3 αCD28 ConA) for 4 h. These data are representative of 1 bird. Technical replicates were carried out in triplicate. No chIL-17F or chIL-2 mRNA expression was detected.

6.4 Discussion

The aim of this Chapter was to differentiate chicken Th17 cells *in vitro* from naïve CD4⁺ T cells using recombinant cytokines, in a similar method to mammals, and measure mRNA levels of the Th17 effector cytokines and the IL-23R to confirm a Th17 phenotype. Ideally, antibody reagents would be available in the chicken, as they are in mammals, to identify Th17 cytokine expression, as well as the IL-23R on the cell surface in chicken cells. Further, mammalian studies use antibodies to inhibit Th1 and Th2 phenotypes at the same time as driving cells towards a Th17 phenotype. The majority of these antibody reagents are not available in the chicken so the established method of measuring mRNA expression by RT-qPCR was used as an alternative.

In vitro mammalian studies use *ex vivo* naïve CD4⁺ T cells, which are activated by plate-bound CD3 and CD28 antibodies (Veldhoen *et al.*, 2006). The optimal concentrations of CD3 and CD28 antibodies for maximal chicken splenocyte proliferation were established. The optimisation assay set out to find out which α CD3 (Southern Biotech or AV36), and what dilution of α CD28, were most effective at stimulating chicken splenocytes. The concentration of α CD3 (1 μ g/ml) was established by previous work within the group. One μ g/ml of CD3 antibody (AV36) and a 1/250 dilution of CD28 antibody (AV7) were optimal for stimulating chicken splenocytes and were used throughout the on-going study.

A negative FACS cell sort, where unstained cells were selected for, was used to purify chicken CD4⁺ T cells from splenocytes. FACS was chosen as the purification method of chicken CD4⁺ T cells as individual cells are able to be purified based on

size, granularity and fluorescence. It is the preferred method of cell sorting when a very high purity of the desired cell population is required. The strategy of negative FACS to derive chicken CD4⁺ T cells was used to avoid activating or stimulating the T cells during the sorting process.

From the total splenocyte population, 7% were CD4⁺ T cells and this cell population had a purity of > 80%. This experiment was repeated three times with similar results. Sorting parameters were adjusted for the requirement of cell purity, not yield, to allow for a more pure CD4⁺ T cell population and not an increased cell number during the cell sort. CD4⁺ T cells make up approximately 15-20% of the total splenocyte population in the J line chicken (Figure 6.4 & Figure 6.6). Therefore, 7% sorted CD4⁺ T cells from the total splenocyte population is less than the typical CD4⁺ T cell percentage in the spleen. This lower percentage of CD4⁺ T cells collected may be because of a loss of CD4⁺ T cells during the sorting process as a result of non-specific antibody staining of these cells. Despite successfully obtaining CD4⁺ T cells post-sort, following pooling and centrifugation of the sorted cell population, there was no cell pellet visible. Following Trypan blue staining to identify live/dead cells, there were no live cells visible under the microscope. It is assumed that the sorted population of CD4⁺ T cells had died between sorting and subsequent centrifugation.

On repeating this experiment, a 9-week old bird instead of a 7-week old bird was used to increase the size of the spleen and, therefore, to increase the yield of splenocytes for cell sorting. The splenocytes were also incubated with the staining Abs at room temperature instead of over ice to investigate if this had a positive effect on cell survival post-sort. Cell viability and yield can be compromised during cell sorting. Viability and recovery can be improved by using a cell-dependent optimal

temperature and by maintaining collection tubes at an optimal temperature (Basu *et al.*, 2010). A similar percentage, purity and number of CD4⁺ T cells were sorted a second time using negative FACS. The cells survived pooling and centrifugation and the presence of live cells were confirmed by Trypan blue staining. The CD4⁺ T cells were counted and then stimulated (1×10^5 cells/well) using recombinant chTGF- β 4 and chIL-6 for 3 days.

The mRNA levels of chIL-2 and chIL-17F and the rRNA of 28S were measured by RT-qPCR. The rRNA 28S is a chicken housekeeping gene, which is expressed by all chicken cells at a constant rate, observed by our group and others in the field (Li *et al.*, 2005). Expression of IL-2 from mammalian T cells indicates the cells have undergone proliferation (Reviewed by Smith, 1984) and chicken splenocytes, following ConA stimulation, also produce IL-2 (Sundick and GillDixon, 1997). Chicken IL-17F was measured as an indicator of a Th17 phenotype as this was the cytokine expressed by IL-23-stimulated chicken splenocytes (Chapter 3). Low mRNA levels of 28S and no chIL-2 or chIL-17F mRNA were measured. The low levels of 28S mRNA confirmed the presence of chicken RNA and indicated that the amount of RNA extracted from the stimulated CD4⁺ T cells was low. This could be a result of too low a starting cell number (1×10^5 cells/well), however, this is comparable to similar assays in humans (Wilson *et al.*, 2007). The normal levels of 28S mRNA measured from stimulated cells is over 30 corrected 40-Ct. Lack of detection of chIL-2 mRNA suggests the cells did not proliferate whereas lack of chIL-17F mRNA expression suggests the CD4⁺ T cells were not driven towards a Th17 phenotype. This experiment was repeated with the same result. The inability of the cells to proliferate and differentiate into Th17 cells is probably due to the low

number of CD4⁺ T cells sorted from the chicken spleen and the reduced viability of the cells following sorting.

Due to the failure of negative FACS to sort chicken CD4⁺ T cells, a positive FACS was attempted. This would allow a comparison of CD4⁺ cell numbers obtained by both methods and allow investigation of the effects of different sorting methods on chicken CD4⁺ T cell survival. It is possible that the sorted CD4⁺ T cells would be activated by the sorting process. However, the advantages of potentially sorting a greater number of viable chicken CD4⁺ T cells outweighed this potential disadvantage.

Following positive FACS, 14% CD4⁺ T cells were obtained from the total splenocyte population and this cell population had a purity of < 38%. There was an increased percentage of CD4⁺ T cells sorted by positive FACS compared to negative FACS but the cell purity was reduced. Sorting parameters for this cell sort were adjusted to increase cell yield, and not purity as before, to allow for collection of a greater cell number and not a higher purity of CD4⁺ T cells. However, a cell purity of < 38% is low.

Following pooling and centrifugation of the sorted chicken CD4⁺ cell population, there was no cell pellet visible. Trypan blue staining confirmed the presence of dead cells only. The likely death of the sorted cell population was investigated by FACS analysis. This confirmed a large (7-fold) reduction in total CD4⁺ T cell numbers from immediately post-sort (38.4%) to after pooling and centrifugation of the sorted cells (4.9%). This confirmed that the majority of the sorted population of CD4⁺ T cells had died between sorting and subsequent centrifugation. Cell death and/or a reduced

cell recovery occurred following both negative and positive FACS. There are a number of accepted methods to improve the viability and recovery rate of cells during sorting, which were adhered to or changed accordingly to improve cell survival of chicken CD4⁺ T cells during FACS. Polypropylene tubes were used for cell processing and sorting through out. Collection tubes were filled with medium containing chicken serum. Initially, cells were kept on ice pre- and post-sort and then subsequently at room temperature to avoid multiple temperature fluctuations. It is possible that an optimal temperature between approximately 4°C and 21°C is required for sorting of chicken CD4⁺ T cells. During the sorting process, tubes containing cells were inverted regularly and collection tubes were processed immediately after they were filled and collected cells re-suspended in appropriate medium. The collection tubes were centrifuged for over 10 minutes to collect the sorted cells. It is unclear what factor/s were adversely affecting chicken T cell viability and subsequent survival.

In an attempt to find a method of cell sorting that did not adversely affect chicken CD4⁺ T cell survival, a positive MACS cell sort was carried out. FACS analysis was used to assess the purity of the MACS sorted CD4⁺ T cells. The live cell percentage was <54% and the sorted CD4⁺ T cell population was < 80% pure. A total of 3.5 x 10⁷ live cells were counted using Trypan blue. The starting cell numbers of splenocytes pre-sort were similar to the cell numbers used for FACS and so the CD4⁺ T cell numbers following MACS were higher. Both FACS and MACS achieved purities of < 80% for sorted CD4⁺ T cells.

The CD4⁺ T cells were stimulated using recombinant chTGF-β4 and chIL-6 for 5 days. As before, the mRNA levels of 28S, chIL-2 and chIL-17F were measured by

RT-qPCR. Again, low levels of 28S were measured but chIL-2 and chIL-17FmRNA was not detected, indicating the CD4⁺ T cells were not stimulated and did not proliferate.

The positive MACS cell sort was repeated and live chicken CD4⁺ T cells were stimulated by a higher concentration of ConA for a shorter period of time to investigate if the sorted cells were capable of being stimulated. Quantitative RT-PCR did not measure chIL-17F or chIL-2 mRNA expression following ConA stimulation for 4, 24 or 72 h indicating the cells were not stimulated and did not proliferate. Based on the unreliable success of sorting chicken CD4⁺ T cells and the subsequent loss of chicken CD4⁺ T cells, it is assumed these cells did not proliferate nor were able to be stimulated and driven towards a Th17 phenotype due to a reduced viability post-sort. CD4⁺ T cells have been successfully sorted from PBMCs in cattle (Flynn and Marshall, 2011) and the bovine CD4⁺ T cells were subsequently driven towards a Th17 phenotype (Peckham *et al.*, 2014). Bovine cells were sorted by positive MACS whereas human CD4⁺ T cells were isolated by two rounds of magnetic bead depletion (Wilson *et al.*, 2007). Chicken cells are routinely sorted by MACS in our group and stimulated for short periods of time (< 1 d) (Rothwell *et al.*, 2012). It is unclear why in this case chicken CD4⁺ T cells were unreliable to sort by several methods and could be stimulated. Several adapted mammalian methods of sorting CD4⁺ T cells have been attempted in these experiments discussed here in the chicken. However, they could not be continued further due to time constraints. It is paramount to investigate further the issues surrounding the sorting of viable chicken CD4⁺ T cells before attempting to differentiate and identify chicken Th17 cells. A

suitable cell sorting method where chicken CD4⁺ T cells reliably survive for further optimisation of Th17 cell differentiation is needed.

Chapter 7 General discussion

7.1 Overall perspective

The aim of this Thesis was to identify Th17 immune responses in the chicken. The key cytokine, IL-23 and its receptor, at the crux of mammalian Th17 immune responses, were crucially missing from the chicken genome. These genes were identified and cloned, and their protein structure investigated. A recombinant protein of chIL-23 was generated and its bioactivity demonstrated. Messenger RNA levels of the Th17 effector cytokines were measured in tissues, stimulated cells and in infection models in the chicken. Finally, *in vitro* differentiation of Th17 cells from CD4⁺ T cells was attempted. These studies indicated the expression of all Th17-related cytokines in the chicken, similar biological activity of chIL-23 to mammalian IL-23, and a potential role of chIL-17A in Marek's disease.

The Th1 and Th2 paradigm proposed by Mosmann and Coffman (1989) has been universally used to explain how hosts elicit different adaptive immune responses to eradicate invading pathogens. Classically, effector CD4⁺ T cells have been divided into two distinct lineages on the basis of their cytokine profiles: IFN- γ producing Th1 cells or IL-4-producing Th2 cells. The chicken mounts Th1 and Th2 immune responses to infection similarly to mammals (Degen *et al.*, 2004; Powell *et al.*, 2009). It is now apparent that mammalian CD4⁺ cells, upon encounter of specific antigen, are activated, expand and differentiate into various Th subsets expressing both distinct cytokine profiles and effector functions (Korn *et al.*, 2009). Th17 cells are one of the newer T helper cell subsets to be described in mammals and are yet to be described in the chicken. Critically, the mammalian Th17 immune response

functions to clear specific types of pathogen not effectively neutralised by Th1 or Th2 immune responses, particularly extracellular bacterial pathogens (Korn *et al.*, 2009). There are a number of important pathogens within the poultry industry and knowledge of the avian immune response to the majority of these is basic (Wigley, 2013). It is important to understand the potential role of Th17 immune responses in the chicken because a better understanding of the avian immune system is key to developing effective poultry disease control measures for the future. Not only will this improve poultry health and welfare but also reduce, and ultimately prevent, zoonotic transmission of disease to humans via the food-chain.

7.2 Cloning and analysis of chIL-23 and the chIL-23R

The presence of IL-23 and the IL-23R genes in the chicken genome indicates conservation of these genes from before the divergence of mammals and birds from a common ancestor over 300 million years ago. Using an EST from discarded data from the chicken genome build, the full-length chIL-23 p19 sequence was mined from the chicken genome. The predicted chIL-23R sequence in the Ensembl database is annotated incorrectly. Using a GenScan prediction, the full-length correct chIL-23R cDNA was identified. Both chIL-23 and its receptor were cloned from RNA from ConA-stimulated splenocytes. Similarly to mammals, chIL-23 p19 is a 180 aa, with the cysteine residue that binds IL-23 p40, and the tryptophan residue that binds the IL-23R, conserved between chickens and mammals (Chapter 3). The chIL-23R encodes a protein of 816 aa and demonstrates conserved synteny with mammals (Chapter 5).

Both molecules were ubiquitously expressed across a number of lymphoid and non-lymphoid tissues in the chicken as shown by RT-qPCR, similarly to other chicken cytokines and their receptors (Gibson *et al.*, 2012; Rothwell *et al.*, 2012; van Haarlem *et al.*, 2009). Primary immune cells stimulated with mitogens associated with the activation and proliferation of T and B cells up-regulated the chIL-23R mRNA levels but did not affect mRNA levels of IL-23 p19 in the chicken. Expression of mammalian IL-23 is primarily from APCs. Therefore, this experiment could be improved by using a more suitable stimulus to drive chIL-23 p19 mRNA expression. LPS induces IL-23 expression in human monocytes, macrophages and DCs (Roses *et al.*, 2008) and also drives expression of IL-23 in zebrafish leucocytes (Holt *et al.*, 2011). TLR2, 3 and 7 agonists augment the expression of IL-23 from human DCs and mouse macrophages (Al-Salleeh and Petro, 2007; Re and Strominger, 2001). These TLR agonists also induce anti-viral responses in chicken macrophages (Barjesteh *et al.*, 2014) and may well be more applicable for stimulating chIL-23 p19 mRNA expression in splenocytes.

7.3 Biological activity of rchIL-23

Mammalian IL-23 is a heterodimeric cytokine composed of a p19 and p40 subunit and is a member of the IL-12 family of cytokines (Oppmann *et al.*, 2000). The full differentiation and maintenance of Th17 cells is dependent on IL-23 (Korn *et al.*, 2009; McGeachy *et al.*, 2009) and it is fundamental to maintain Th17 cells *in vitro* and *in vivo* (Chen *et al.*, 2011). IL-23-stimulation of splenocytes drives expression of IL-17A and IL-17F (Aggarwal *et al.*, 2003). Many *in vitro* and *in vivo* studies have investigated the biology of IL-23 in human and rodent systems, the findings of which

are not always applicable to cytokine function in other species, particularly the bird. However, mRNA levels of chIL-17A, chIL-17F and chIL-22 were up-regulated in splenocytes stimulated with rchIL-23, demonstrating a bioactivity of rchIL-23 similar to mammals, and the evolutionary conserved functional importance of this molecule.

7.4 Th17 cytokines in the chicken

Th17 immune responses in mammals are mediated by the production of pro-inflammatory effector cytokines such as IL-17A, IL-17F, IL-21 and IL-22, resulting in protection from invading microbial pathogens (Korn *et al.*, 2009). The Th17 cell subset has not yet been described in birds but several components of Th17 immune responses have been identified in the chicken including the main effector cytokines chIL-17A, chIL-17F, chIL-21 and chIL-22. Expression of these cytokines was investigated in tissues, cells and during infection models in the chicken. Chicken Th17 mRNA levels were detected in nearly all tissues sampled although at low levels. It is tempting to speculate that expression of these cytokines in such a wide variety of tissues suggests that Th17 cytokines are more widely expressed in the chicken than in mammals. However, it should be noted that RT-qPCR is a sensitive technique. Also, as discussed previously, the high mRNA levels seen in non-lymphoid tissues may in part be explained by the chicken's lack of lymph nodes and their development of diffuse lymphoid aggregates in organs (Michael and Hodges, 1974). Consequently, tissues defined in mammals as non-lymphoid contain more lymphoid cells in the chicken. Further, it must be borne in mind that expression of mRNA does not necessarily correlate with expression of functional protein. As

discussed previously, the ubiquitous expression of cytokines and their receptors in lymphoid and non-lymphoid tissues, using a sensitive technique such as RT-qPCR, is a common finding in the chicken. As such, this work is highly suggestive of the chicken mounting Th17 immune responses similarly to mammals and provides the basis for further investigation of Th17 cytokine expression in the chicken, eliciting which cells express Th17 cytokines under specific conditions *in vitro*.

Primary immune cells in the chicken stimulated with mitogens generally up-regulated expression of the chicken Th17 effector cytokines, with levels decreasing over time. These data suggest the chicken Th17 effector cytokines may be expressed during Th17 immune responses in the chicken. This is the first time Th17 immune responses have been studied in the chicken, therefore, the potential role of clearing infection not neutralised by Th1 or Th2 immune responses also required investigation. Expression of the Th17 cytokines was investigated during infection in the chicken, using archived samples. In chickens infected with MDV, an alphaherpes virus that drives Th1 immune responses in the chicken, susceptible line 7₂ birds expressed higher levels of chIL-23 p19, chIL-17A and chIL-21 mRNA compared to resistant line 6₁ birds, suggesting an early involvement of IL-17 immune responses in susceptibility to MDV infection (chapter 4). Of the pathogens in mammals known to drive Th17 immune responses and, therefore, require a Th17 adaptive response to overcome infection, *S. aureus* infection is currently the only disease model established in poultry. In an experimental BCO model, higher levels of chIL-17A mRNA were measured in the bone marrow of the wild-type and mutant groups at 6 dpi compared to uninfected controls, suggesting the possibility of a local chIL-17A response to infection. These preliminary studies validate the need for further

investigation of Th17 immune responses in the chicken and provide a basis from which to do this.

7.5 Future experiments

7.5.1 Further understanding of the role of chIL-23

The discovery of mammalian IL-23 and the revelation of the biology that this cytokine governs, led to significant new insights within mammalian immunology (Cua *et al.*, 2003; Langrish *et al.*, 2005; Oppmann *et al.*, 2000). IL-23 plays crucial roles in orchestrating innate and adaptive immune responses and in the pathogenesis of mammalian autoimmunity (Reviewed by Gaffen *et al.* (2014) and Sutton *et al.* (2012)). Now that the IL-23 p19 subunit has been identified in the chicken, more detailed studies of its role in the avian immune response and in disease models is possible as well as a comparison of its role in birds compared to mammals. Recent work has shown that Th17-like responses appear to take place within a zebrafish autoimmune model, indicated by an increase in IL-17 expression, and provide evidence that cytokines such as IL-23 are involved in a non-mammalian species (Quintana *et al.*, 2010). The development of a mAb, in conjunction with the biologically active recombinant protein of chIL-23, would provide reagents to fully understand the cells and tissues which express chIL-23. Having the chIL-23 p19 subunit, along with many of the important cytokines that are expressed by different T cell subtypes, will aid future investigations into the types of Th and Treg cells that exist in birds.

7.5.2 The *chIL-23R* and *chROR γ t* as potential Th17 cell markers

T helper cells in mammals, classified as Th1, Th2 or Th17, are identified by expression of specific transcription factors and by the cell surface expression of specific receptors. Mammalian Th17 cells express IL-23R and it is used *in vitro* and *in vivo* to identify Th17 cells (Wilson *et al.*, 2007). Having identified the IL-23R in the chicken, development of a *chIL-23R* mAb would further this study by identifying cells in the chicken which express this mammalian Th17 cell surface marker. It would be worth generating mAbs against *chIL-23* and the *chIL-23R*, because IL-23 and its receptor are so important for Th17 immune responses in mammals, and most of the biological roles of IL-23 and IL-23R in mammals were demonstrated by anti-IL-23 and anti-IL-23R mAbs. ROR γ t is the master regulator of transcription for mammalian Th17 cells but is not in the current build of the chicken genome. Identification of this transcription factor in the chicken would provide another tool with which to potentially identify chicken Th17 cells.

7.5.3 Understanding the role of Th17 immune responses in the chicken

This Thesis investigated the expression of Th17 effector cytokines in disease models already established as driving Th1 or Th2 immune responses in the chicken. From this preliminary data, it is clear that there are Th17 immune responses in the chicken, and these Th17 cytokines are possibly expressed during infections previously assigned as generating Th1 or Th2 immune responses. However, the role of Th17 cytokines during infection in the chicken remains unclear. A further MD experiment was planned, which involved a longer time course, larger group sizes and additional tissue sampling, to study Th17 effector cytokine mRNA levels during MDV infection. Unfortunately, there was an outbreak of MD within the unit where the

experiment was planned and the study did not go ahead. Whilst research on the pathogenesis of MD has come a long way, there are details which remain elusive and the potential involvement of Th17 immune responses may highlight methods to manipulate the immune response for better protection against the disease.

The *S. aureus* pilot study, whilst remaining inconclusive about Th17 involvement, suggests the potential role of Th17 cytokines also needs further study. *S. aureus* is known to drive Th17 immune responses in mammals and the results in the chicken imply the same may be true. Once an infective dose of *S. aureus* is established, increasing the number of birds, including earlier time points for sampling and sampling more tissues, would allow for a more thorough investigation of Th17 cytokine expression during *S. aureus* infection.

7.6 Conclusions

In summary, IL-23 and the IL-23R, which are vital to Th17 immune responses in mammals, are present in the chicken. IL-23, a mammalian cytokine required for full differentiation and maintenance of Th17 cells, shows similar biological activity in the chicken. Th17 effector cytokine genes are expressed in stimulated lymphoid cells and during infection of the chicken. These data confirm that there are Th17 immune responses in the chicken. The study of chIL-23, the chIL-23R and the Th17 effector cytokines in the chicken, as well as the reagents and methods established in this project, provide useful tools and will aid further investigation of Th17 immune responses in the chicken.

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Appendix 1 Buffers and solutions

QIAprep® spin miniprep kit (Qiagen)

Buffer P1 (dissolving buffer)	50 mM Tris-Cl (pH8.0), 10 mM EDTA, 100 µg/ml RNase A
Buffer P2 (lysis buffer)	200 mM NaOH, 1% SDS (w/v)
Buffer N3 (neutralising buffer)	contains guanidine hydrochloride
Buffer PB (binding buffer)	contains guanidine hydrochloride
Buffer PE (wash buffer)	contains guanidine hydrochloride

EndoFree® plasmid Maxi kit (Qiagen)

Buffer P1 (dissolving buffer)	50 mM Tris-Cl (pH 8.0), 10 mM EDTA, 100 µg/ml RNase A
Buffer P2 (lysis buffer)	200 mM NaOH, 1% SDS (w/v)
Buffer P3	3 M potassium acetate (pH 5.5)
Buffer ER	contains isopropanol and polyethylene glycol octylphenyl ether
Buffer QBT (equilibrium buffer)	750 mM NaCl, 50 mM MOPS (pH 7.0), 15% isopropanol (v/v), 0.1% Triton®X-100
Buffer QC (wash buffer)	1 mM NaCl, 50 mM MOPS (pH 7.0), 15% isopropanol (v/v)
Buffer QN (elution buffer)	1.6 mM NaCl, 50 mM MOPS (pH 7.0), 15% isopropanol (v/v)

QIAquick PCR purification kit (Qiagen)

Buffer PB (binding buffer)	contains guanidine hydrochloride and isopropanol
Buffer PE (wash buffer)	contains ethanol

QIAquick gel extraction kit (Qiagen)

Buffer QG (dissolving buffer)	contains guanidine hydrochloride
Buffer PE (wash buffer)	contains ethanol

RNeasy mini kit (Qiagen)

Buffer RLT (lysis buffer)	contains guanidine hydrochloride
Buffer RW1	contains guanidine hydrochloride
Buffer RPE (wash buffer)	contains ethanol

Serum-free growth DMEM

DMEM

1% 200 nM L-glutamine

1% 100X Non-essential amino acids

1% 100X Sodium pyruvate

Growth DMEM

DMEM

10% foetal bovine serum

1% 200 nM L-glutamine

1% 100X Non-essential amino acids

1% 100X Sodium pyruvate

Complete DMEM

DMEM

0.5% BSA

1% 200 nM L-glutamine

500 µl 1000X Penicillin/streptomycin

Complete RPMI

RPMI

10% Chicken serum

1% 200 nM L-glutamine

500 µl 1000X Penicillin/streptomycin

FACS buffer

PBS

0.5% (w/v) BSA

MACS buffer

PBS

0.5% (w/v) BSA

0.4% 0.5 mM EDTA

Blocking buffer

PBS

0.2% (w/v) casein

Appendix 2 Vectors

pGEM®-T Easy

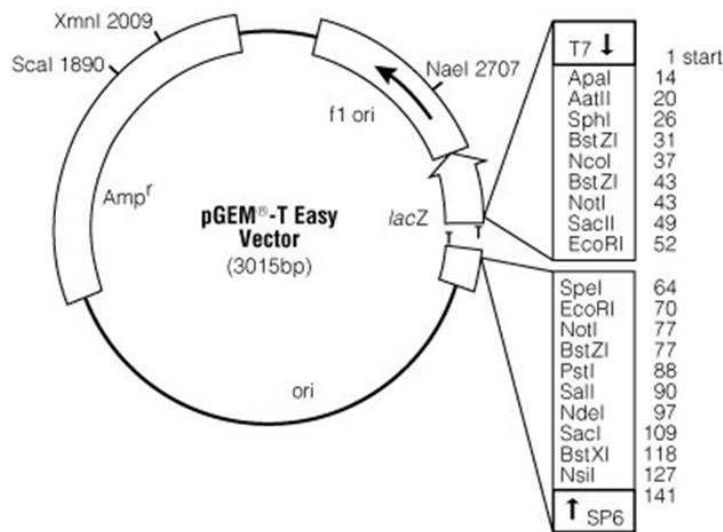


Figure 7.1. pGEM®-T Easy plasmid map.

pCI-neo

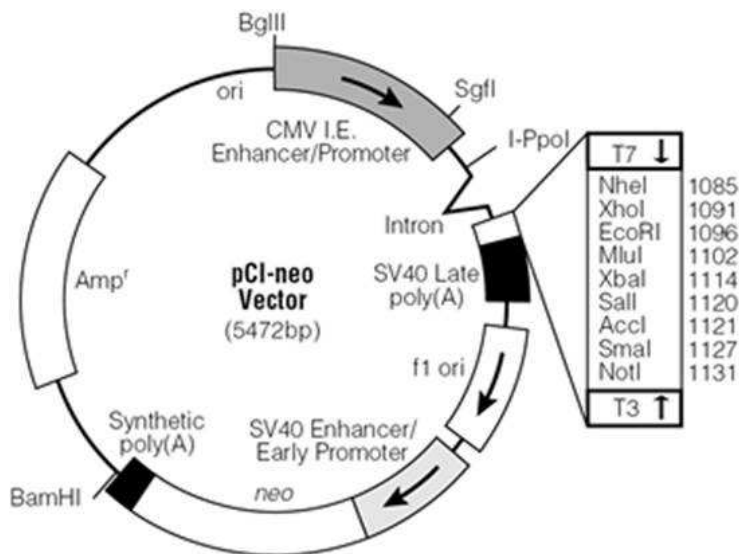


Figure 7.2. pCI-neo plasmid map.

pTarget™

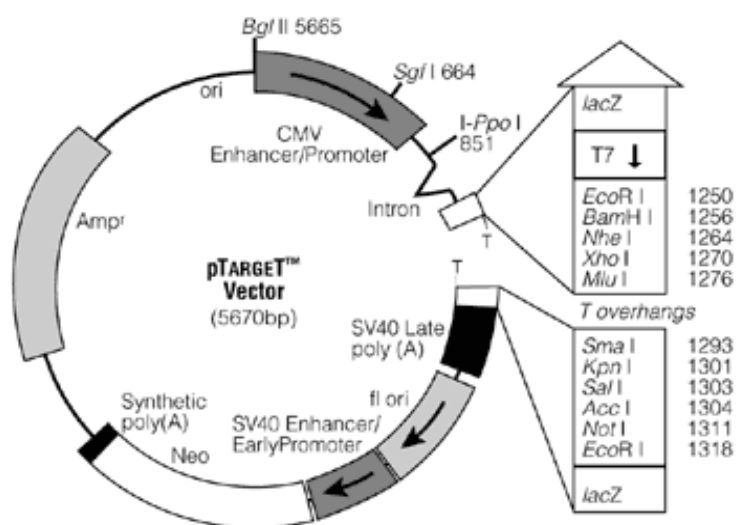


Figure 7.3. pTarget™ plasmid map.

pcDNA3

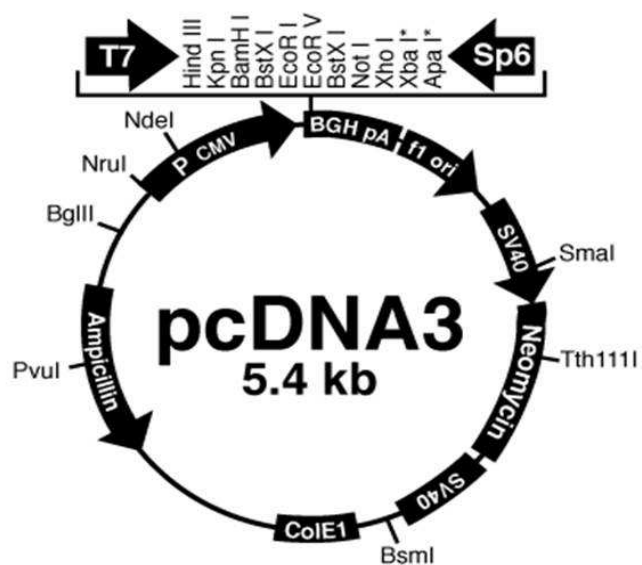


Figure 7.4. pcDNA3 plasmid map.

pcDNA3.1(+)

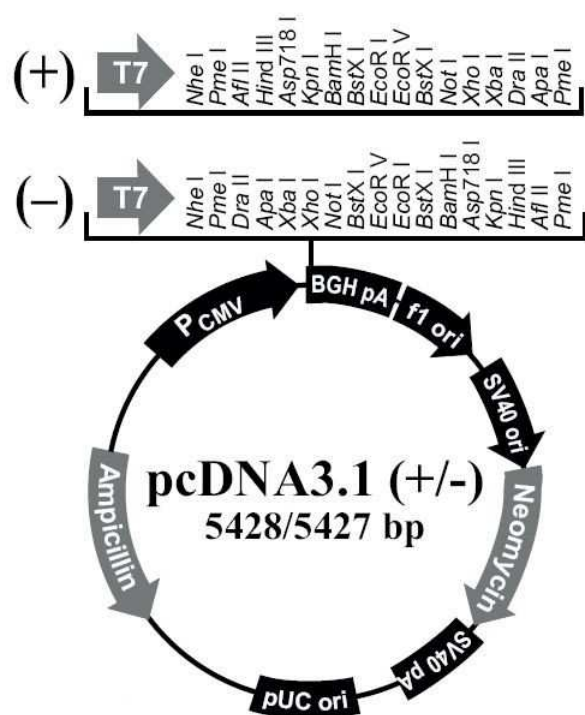


Figure 7.5. pcDNA3.1 plasmid map.

pKW08

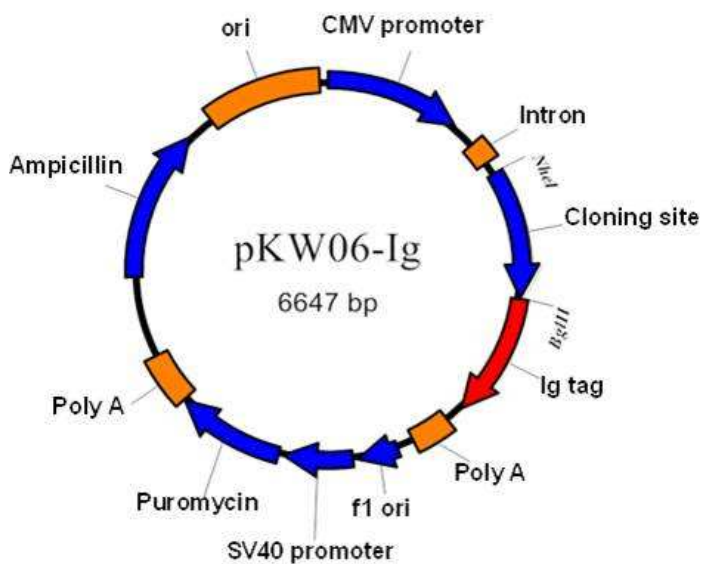


Figure 7.6. pKW06-Ig plasmid map.

Appendix 3 Cells, recombinant protein and antibodies

Bacterial strains

Escherichia coli competent cells, JM109 strain cells: e14⁻(McrA⁻) *recA1 gyrA96 thi-1 hsdR17* (r_k⁻m_k⁺) *supE44 relA1 Δ(lac-proAB)* [F' *traD36 proAB lacI^qΔM15*] (Stratagene 200235) were used to clone plasmid DNA.

COS-7 cells

The COS-7 cell line is a fibroblast-like cell line derived from African green monkey kidney tissue. The COS-7 cell line was derived by immortalising CV-1 cells with a defective mutant of the SV40 virus, which can produce large T antigen but has a defect in genomic replication itself. It is an adherent cell line that can be transiently transfected for high level expression of recombinant protein (Gluzman, 1981).

Recombinant protein

Table 7.1. Recombinant proteins used in this study.

Cytokine	Vector
IL-2	pCIneo
IL-4	pCIneo
IL-6	pKW08
IL-7long	pcDNA3.1
IL-12β	pCIneo
IL-12	pcDNA3.1
IL-17A	pCIneo
IL-17F	pTargeT
IL-21	pCIneo
IL-22	pCIneo
IL-23	pcDNA3.1
IL-23R	pTargeT
GM-CSF	pCIneo
TGF-β4	pCIneo

Antibodies

Table 7.2. Antibodies used in this study. ch = chicken; m = mouse.

Antigen	Species	Clone/Subclass	Conjugation	Supplier/Source
chCD3	Mouse	AV36/IgG1	Unconjugated	T.F. Davison (Institute for Animal Health, Berkshire, UK)
chCD28	Mouse	AV07/IgG1	Unconjugated	Young <i>et al.</i> , 1994)
chTCR1	Mouse	IgG1	FITC	Southern Biotech
Chicken monocytes/macrophages	Mouse	KUL01/IgG1	FITC	Southern Biotech
chCD8 α	Mouse	IgG2b	PE	Southern Biotech
chBu1	Mouse	AV20/IgG1	AF647	Southern Biotech
chCD4	Mouse	CT-4/IgG1	Unconjugated	Southern Biotech
chCD4	Mouse	CT-4/IgG1	PE	Southern Biotech
chIL-12p40	Mouse	AV134	Unconjugated	Balu <i>et al.</i> , 2011)
chIL-12p40	Mouse	AV135	Unconjugated	Balu <i>et al.</i> , 2011)
chIL-12p40	Mouse	AV136	Unconjugated	Balu <i>et al.</i> , 2011)
mIgG1	Goat	IgG1	HRP	Southern Biotech

Appendix 4 Chicken IL-23R primers

1 ATGTTTCGTGCAAAGAAAAAGCAGCAAACGTGCCCCGCCCCATCGCAGTACCTGCCCTCC
1 -M--F--R--A--K--K--K--A--A--N--V--P--G--P--S--Q--Y--L--P--S--

61 CCCCCGACCGCCCCGGCCCGGCTGCTTCGTTTCGGGGAAATCCCGCCCCAGAAGCGGCTG
21 -P--P--D--R--P--G--P--A--A--S--F--G--E--I--P--P--Q--K--R--L--

121 TTTGCGGTGCCTCCTTCCCGCCCCGGGCGCAGTGCCCGCTCCGGCCCTTCCTTCTGTCTG
41 -F--A--V--P--P--S--R--P--G--R--S--A--R--S--G--P--S--F--C--L--

181 CTCCGCGGGGCTCTCAGCTCCCAGTTCACCAAAAGTAACAAAATCCCCGATTTCACCCC
61 -L--R--G--A--L--S--S--Q--F--T--K--S--N--K--I--P--R--F--H--P--

241 ATAGGGTGGGAGGTGTGCGGGCAGCTGGGAGGCCTTCAGAGCCCCTCCCTGGGTGCGTTT
81 -I--G--W--E--V--C--G--Q--L--G--G--L--Q--S--P--S--L--G--R--F--

301 GCGCCTGGCTGTTCTTATGAACGCTTTCAGTGTGCTGAAGCAAATGAGGTCAGCGCA
101 -G--A--W--L--F--L--M--N--A--F--T--V--L--E--A--N--E--V--S--A--

361 CACATCCCGAAGGCTATGCCCACTGCCACGCAGGCAGCAGCCGCAGCCAGGCCATG
121 -H--I--P--K--A--M--P--H--C--P--T--Q--A--A--A--A--A--Q--A--M--

421 GTGGGAGGCGGAGAGGCTTTCTGTGCTCCATATCCTGCTCTGCATTCTGGGCAGAGGTGCT
141 -V--G--G--G--E--A--F--V--L--H--I--L--L--C--I--L--G--R--G--A--

481 GCCCAAATCAGGTGCCTGGGGCACGTGAGGATCGAGCCGGGCTCCGTGGTGCCAAATGGGT
161 -A--Q--I--R--C--L--G--H--V--R--I--E--P--G--S--V--V--P--M--G--

541 TCCAACGTCTCCATCACTGTGTCTCGGCGCTGGACTGCCCGTGGGCGAGCCTCTCCATC
181 -S--N--V--S--I--T--C--V--S--A--L--D--C--P--W--A--S--L--S--I--

601 CTGCTCAACCTCACAGCCCCAAGGATCCCCACGCCCTCTCAACAGCAGTGCAGCCAG
201 -L--L--N--L--T--A--P--K--D--P--P--R--P--L--N--S--S--A--A--Q--

661 CTGCAGCTGCGTGGTTTCCGCCTGCCCTTCAGCATCATCACCTGCCTCGCCCGCTGCCCC
221 -L--Q--L--R--G--F--R--L--P--F--S--I--I--T--C--L--A--R--C--P--

721 AACAGCTACTGGAACGAGGTGGTCTCGGGACGGAGCTCTGGGCCGGCTACCCCCGGGC
241 -N--S--Y--W--N--E--V--V--C--G--T--E--L--W--A--G--Y--P--P--G--

781 CCCCCACCAACCTGAGCTGTGCCATCCCCGAGCGCTCAGGGAGCCTGGCATGCACATGG
261 -P--P--T--N--L--S--C--A--I--P--E--R--S--G--S--L--A--C--T--W--

841 GACGCCGGGCGAGCAACACTGCTGCACACCAACTACAGCCTCCACCTGCGCAGCATACAG
281 -D--A--G--Q--P--T--L--L--H--T--N--Y--S--L--H--L--R--S--I--Q--

901 ACGGCACAGGAGGATGTGTTCCCCACGGGCTCACCAGTGCCCTTGAGTGCTCTGAATGGC
301 -T--A--Q--E--D--V--F--P--T--G--S--P--V--P--L--S--A--L--N--G--

961 AGCAGTCGCTACCTGGCATGGGTGCAGGCCAGCAATGCACTGGGCATGGCACGCTCAGCC
321 -S--S--R--Y--L--A--W--V--Q--A--S--N--A--L--G--M--A--R--S--A--

1021 CCTCTGCACCTCGACCTGCAGCAGCTCGTGGTGCCCGCTGCCCTTGGCTGAGAGCGCA
341 -P--L--H--L--D--L--Q--Q--L--V--V--P--A--V--P--L--A--E--S--A--

1081 GAGACGACGGCGGGGTACCTCCCACCACCACCATCCGTTGGCGACAGCAGACAGAAGT
361 -E--T--T--A--G--S--P--P--T--T--T--I--R--W--R--Q--Q--T--E--L--

1141 CAGGACGTGCGCTGCGAGGAGCGGCACAAGGCAGTGGGTGCTGCGGAGTGGCATGTGACA
381 -Q--D--V--R--C--E--E--R--H--K--A--V--G--A--A--E--W--H--V--T-

1201 GCATGGGACAGTGCAGTGCAGCGTGGACACCAGCTTCAGAGTGCCACCCGCTACGTGTTC
401 -A--W--D--S--A--V--Q--R--G--H--Q--L--Q--S--A--T--R--Y--V--F-

1261 CAGGCACGGTGCCGGCTCAGCGCTGCAGGCAGCCCTGGAGTGCTTGGGGCTGCCCTTC
421 -Q--A--R--C--R--L--S--A--A--G--S--P--W--S--A--W--G--L--P--F-

1321 ACCTACAGCACACCCGAGGCTGCCCCGCGGCAGCACCCGACGTGTGGCGGCGGCTGGGG
441 -T--Y--S--T--P--E--A--A--P--A--A--A--P--D--V--W--R--R--L--G-

1381 CGGCTGTTCCCCAACGGCAGCCACGAGGTGACAGTCTTGATCAAGCCGCCCCGCGATGCC
461 -R--L--F--P--N--G--S--H--E--V--T--V--L--I--K--P--P--R--D--A-

1441 CGCACGCCCATCCTGGGCTACGCCGTGTGGGCTGGAGCCTCGCGTCCCCTCTGCAATACA
481 -R--T--P--I--L--G--Y--A--V--W--A--G--A--S--R--P--L--C--N--T-

1501 TCGAGCACCGCCTGCAGCCTCCTCCTGCCTCCGGCACTGCGCGACCTCCGCGTCACCGCC
501 -S--S--T--A--C--S--L--L--L--P--P--A--L--R--D--L--R--V--T--A-

1561 TACAACGCCCCGGGGGGCTTCGAGCCCCGCGGTGTGACGCTCCGCCGGGACCCCCAGCCCCG
521 -Y--N--A--R--G--A--S--S--P--A--R--V--T--L--R--R--D--P--S--P-

1621 GAAGCCTTCCCATCACCAGTGGCTGTGGATGTGCAGCGTGAGAACCAGAGCACCATATCT
541 -E--A--F--P--S--P--L--A--V--D--V--Q--R--E--N--Q--S--T--I--S-

1681 GTGGCCTGGCAGCCCCCCCCGGCACAACGGGACCCCCCACTCTGGTTCATAGTGGAGTGG
561 -V--A--W--Q--P--P--R--H--N--G--T--P--P--L--W--F--I--V--E--W-

1741 GTTTGTACAGCTCCGTACAGCCATGAGGAAGAGTTCTTTTGAAGAAAGTCCCAGGCCAG
581 -V--C--T--A--P--Y--S--H--E--E--E--F--F--W--K--K--V--P--G--Q-

1801 GACAGCCACACCTACATCCAAGAGGACGCAGCAGCAGGAAGCCACATCAACGTGTCGGTG
601 -D--S--H--T--Y--I--Q--E--D--A--A--A--G--S--H--I--N--V--S--V-

1861 TATGCAGTCTACCCAAATGGAGTGAGCAAACCAAGCTCTGGCCAAGTTTCTTTGGAGGAC
621 -Y--A--V--Y--P--N--G--V--S--K--P--S--S--G--Q--V--S--L--E--D-

1921 CAGATGTTGGGCATTATCTACCCAGAGGCTTTGCATGACCATCAGAACTGTGAGGGTAAA
641 -Q--M--L--G--I--I--Y--P--E--A--L--H--D--H--Q--N--C--E--G--K-

1981 CCTGTAGGGACAGCAACAGAAATGCAAGCAGTTGGGTTCAGCATGACTGACTGTGGATGC
661 -P--V--G--T--A--T--E--M--Q--A--V--G--F--S--M--T--D--C--G--C-

2041 AGAATTATTACAAAATTAAGGCAGCAGTGGCACTGGTTTTGCCAAAATGGCTGCTTGAA
681 -R--I--I--H--K--I--K--A--A--V--A--L--V--L--P--K--W--L--L--E-

2101 GATTTCCCCACATGGAAAACAGCAAGGTGATAAAGTCCTTCCAGGAGAAGAGTGAATTC
701 -D--F--P--H--M--E--N--S--K--V--I--K--S--F--Q--E--K--S--E--F-

2161 ACAAACAGCATTTTTCAGTGAGCCGTTCTGGATAACAGTGACCCCAATACGGAAATA
721 -T--N--S--I--F--S--E--P--F--L--D--N--S--D--P--T--I--T--E--I-

2221 AAGGAGCTGTGAGGGGACAAGAAATACAAGACGTGGACATCAAAAAGGAACCCAGCAAT
741 -K--E--L--S--G--D--K--K--Y--K--N--V--D--I--K--K--E--P--S--N-

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2281 GTGGTCCCTGAGAACGTGGAGCACCCACAGAGCTCAGCACCTCCCCATAACACGGCCACG
761 -V--V--P--E--N--V--E--H--P--Q--S--S--A--P--P--H--N--T--A--T-
      ←
2341 GAGGACGTCAGTGACTACAAACCCCAAATCTCTGATGCAAACACACTAGGATATGTAGCT
781 -E--D--V--S--D--Y--K--P--Q--I--S--D--A--N--T--L--G--Y--V--A-

2401 GCCAACATGGGCCTGATACAGCCCTACACACCAGCTCCAGAACCAGAGACAAATATTTTC
801 -A--N--M--G--L--I--Q--P--Y--T--P--A--P--E--P--E--T--N--I--F-

2461 TTCCGAGACTACAGCAGCCCTTTTAGCTGCCTGTGGGATGCTGAAGGAGCAGGGGCCAG
821 -F--R--D--Y--S--S--P--F--S--C--L--W--D--A--E--G--A--G--A--Q-

2521 GTGTGCCTGCTGGACAAGATCAACCTGGTTTTAAACAACGACAGAAGTGGACAAAACCAC
841 -V--C--L--L--D--K--I--N--L--V--L--N--N--D--R--S--G--Q--N--H-

2581 GCATTTCAGCTCCGCCCAGGAGGAACAAAATGCCCTTTTGGAAAACAGTGGGAAAAACA
861 -A--F--S--S--A--Q--E--E--Q--N--A--L--L--E--N--Q--W--E--K--T-
      ←
2641 CTGTACAGTGAAGGAGTTCAGGAACAAACCTTGGTTCCAGATGAGCTGGTTTCTTGCTTA
881 -L--Y--S--E--G--V--Q--E--Q--T--L--V--P--D--E--L--V--S--C--L-
      ←
2701 AGGGCAATGAATGGAGGATCTGTGGATATACAGACCTGCTTTCCCCAGAGCATCGGAAGA
901 -R--A--M--N--G--G--S--V--D--I--Q--T--C--F--P--Q--S--I--G--R-
      ←
2761 TTGTTTTGA
921 -L--F--*-

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Figure 7.7 Predicted coding nucleotide sequence (2,769 nt) and amino acid sequence (922 aa) of chicken IL-23R (GENSCAN00000023581). Primers designed are underlined. Primers used for amplification of chIL-23R cDNA sequence in colour: IL-23R F10 green and IL-23R R1b in blue. Primers in red did not amplify cDNA sequence. Primer orientation indicated by arrows above nucleotide sequence.

Table 7.3. Primer sequences for PCR amplification of chIL-23R

Primer name	Sequence (5'-3')
IL-23R_F1	ATGGAAAACAGCAAGGTGATAA
IL-23R_F2	AAATGGCTGCTTGAAGATTTC
IL-23R_F3	ATCAGGTGCCTGGGGCA
IL-23R_F4	GTTCGGGGAAATCCCGCCCC
IL-23R_F5	ATGGGTCCAACGTCTCCATCAC
IL-23R_F6	GAGGTGGTCTGCGGGACGGAG
IL-23R_F7	CTGCGCAGCATACAGACGGC
IL-23R_F8	CTGCAGCAGCTCGTGGTGCC
IL-23R_F9	TGGCATGTGACAGCATGGGAC
IL-23R_F10	ATGAACGCTTTCCTGTGCTTGAA
IL-23R_F11	ATGCCCCACTGCCCCACGC
IL-23R_F12	ATGGTGGGAGGCGGAGAGG
IL-12R_F13	ATGTTTCGTGCAAAGAAAAAAGCAG
IL-23R_R1	TCAAAACAATCTTCCGAGCTC
IL-23R_R1b	TCA AAA CAA TCT TCC GAT GCT CTG
IL-23R_R2	ACGCTGCACTGCACTGTCCC
IL-23R_R3	TAAGCAAGAAACCAGCTCAT
IL-23R_R4	AGAGATTTGGGGTTTGTAGTCAC
IL-23R_R5	GGCCTGCACCCATGCCAGG
IL-23R_R6	GAAATCTTCAAGCAGCCATTT

Appendix 5 Chicken IL-23R gene structure

>chromosome:Galgal4:8:27528853: 27541472

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27528853 GAGCCCCCTCCCTGGGTTCGGTTTGGCGCCTGGCTGTTTCCTTATGAACGCTTTCACTGTGCT 27528912
27528913 TGAAGCAAATGAGGTCAGCGCACACATCCGAAGTAACAGGAGGAGAAAGGCAGAGGTG 27528972
27528973 TTAGCTGCATCTGGGAGGCTGCTTCGGGATGGATAATGCTGCTCTGAAGCTTCTTGAAGC 27529032
27529033 TTGGGGTGGATAATGCTCTTTTGAAGCTTTTGCAGTTGATTCTGTGCACCGTTCCCAAAA 27529092
27529093 GGAGCCCTTCATCAGTGCCCCGAGTGGGGTTTCCCCAGGGCCAGCGGGTGCAGTGGGAT 27529152
27529153 GGGAGATGTGAGCCGGGTGGGAAGGAGTGAAGTGAAGGGAGTGCAGGGAACCTCAGGAGA 27529212
27529213 GCCTGGGGACGTGGCCATGAGCTTGTGTCTTGGAGGGCTGGGAAAGCCCTGCTGGGCC 27529272
27529273 TGAGGGGCAGCAGCTGAAGCACAGTGGGTGGGGACGTGAGAAAGTTTGGTGTTCCTCAT 27529332
27529333 CCCATGGCACCACAGCCATGTGGGTTTGGCCATTTCCAGGGATGGAGGCTCCACGGCAGC 27529392
27529393 CCACTCGCAAGCTCTGGCACCTTTATGGCAAAAATATTTTCTTGAAGTTTCAATTTTGG 27529452
27529453 TCTCCATCCCAGCTGTGCAGCCACTGCTCCATCAGATCTCTGTCCTTGGCATACTGGGCA 27529512
27529513 TGGGGGTGGCTCCCCAGTGTCCTCCGAGTCCCCCAAGCACCCAGGCTGGGTGCCTGGCA 27529572
27529573 GCACCGACTCCTTCCTTCCTCCCTAGGCTATGCCCCACTGCCCCACGCAGGCAGCAGCCG 27529632
27529633 CAGCCCAGGCCATGGTGGGAGGCGGAGAGGCTTTCGTGCTCCATATCCTGCTCTGCATTC 27529692
27529693 TGGGCAGAGGTAAGTGGCCATTCAGCATGGCTTGGCACAGCACGTGATGGCATAGCTGTG 27529752
27529753 CCTTTTCCAGGAAAGCCAAATCGAGCTGATGTGTCATTTGAGCATGAGCTTTATCAGTGCTG 27529812
27529813 CTGCAGAGCCCCAATCTGATAGCGGAGTCAAGTGCAGTGCCTGTAATCTCAGGGATCAGAG 27529872
27529873 CTTTCAGCCTATGCGTTATTTCCCTGCAGTCATTTAAGCATTAGAGCAAAAGGTAAAATC 27529932
27529933 AGTGAGGCTGTTTCCGAGCTGAGCAATGAGTACCGGCTTCTGGCACGGGGTTTACCCTG 27529992
27529993 CACTTGGGGGGCTGCATTGAAGCTGCGAGCATTGTGAGGTGCTGCCCAAATCAGGTGCC 27530052
27530053 TGGGGCACGTGAGGATCGAGCCGGGCTCCGTGGTGCCAATGGGTTCCAACGTCTCCATCA 27530112
27530113 CCTGTGCTCTCGGCGCTGGACTGCCCCGTGGGCGAGCCTCTCCATCCTGCTCAACCTCACAG 27530172
27530173 CCCCAAGGATCCCCACGCCCTCTCAACAGCAGTGCAGCCAGCTGCAGCTGCCTGGTT 27530232
27530233 TCCGCCTGCCCTTCAGCATCATCACCTGCCTCGCCCGCTGCCCCAACAGCTACTGGAACG 27530292
27530293 AGGTGGTCTGCGGGACGGAGCTCTGGGCCGGCTGTGAGTACTGCCGTGGGGTTACGGGTG 27530352
27530353 ATAGGGGAGCGGTGGGGCTGAAGGCGGGGATGCTGATGCTGCCTCTCTGAGACTCCCCG 27530412
27530413 GACCCCCCACCAACCTGAGCTGTGCCATCCCCGAGCGCTCAGGGAGCCTGGCATGCACA 27530472

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Appendices

27530473 TGGGACGCCGGGCAGCCAACTGCTGCACACCAACTACAGCCTCCACCTGCGCAGGTAG 27530532

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27530593 ACCACAGTGATCATCTTGTTCACCCCCCTGCTATGTACAGGGTCTCCAACCACCAGCCC 27530652

27530653 AGGCTGCCCAGAGCCACATTCAGCCTGGCCTTGAATGCCTCCAGGGATGGGGCATCCACA 27530712

27530713 GCCTCCTTGGGCATCCAGCACCCAGGACACAGCAACCCCATTCAGCATGAACTTAATG 27530772

27530773 GTGCCCAGCACCTAAACAAAAATGCCACCAGGAAATAAGGATTGGTACACAGTGGCCT 27530832

27530833 TCCTGCCCTAGCCCTACATGTGCTGTGCCACATGCAAGGCCAAGCCCCATGGCGTGAGG 27530892

27530893 CTGCTGGGCTTCCCTAGTCAACCCAACTCCGCTATTCAATTACGTGCAGTCTGTGTGGGT 27530952

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27531013 GAGCTGTGGGAGCTCCCTTCCTGCAGCAGAAACGTGAATGCCATGCAGCAGTGCAGGAA 27531072

27531073 CCAAAGAGAATGATTGTCTAGTTCACTTGAAGAGTGAAAAACTGCAGCTCTGGAGATGAC 27531132

27531133 TGTGCCCCATGCGGGGCTCTGAGCATGGCCGCTTCTGCCCCGCGAGCATACAGACGGCAC 27531192

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27531253 GCTACCTGGCATGGGTGCAGGCCAGCAATGCACCTGGGCATGGCACGCTCAGCCCCCTCTGC 27531312

27531313 ACCTCGACCTGCAGCAGCTCGGTACGCCCGTGGCACACACGCTGTGCCCTGCACTGGGGC 27531372

27531373 TCCAACCATGAGCCCTCCAGCACTCTGTGCCCACACGGTGCTGTGACGCCTGGCTGATG 27531432

27531433 GCTGCTGCTCTCCCCACAGTGGTGCCCCCGGTGCCCCCTGGCTGAGAGCGCAGAGACGACG 27531492

27531493 GCGGGGTCACCTCCCAACCACCACCATCCGTTGGCGACAGCAGACAGAACTGCAGGACGTG 27531552

27531553 CGCTGCGAGGAGCGGCACAAGGCAGTGGGTGCTGCGGAGTGGCATGTGATGGCGCTGA 27531612

27531613 GCCCCAGAGGTCCATTATAGCTATGGGTGGGGTCCCCAGGGAGCAGGGCGGGTTGAAC 27531672

27531673 AGTCCTTCCAGCAGCATTATCTCTGCTTTTAAAGCAAAAAGCAAAATTTGTAATATTTGT 27531732

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27531913 ACTCAGCAGAGCTGCCAAAACGGGAGACTGCCTGGTGCCACTGGCACAGAGCTCTGCAGT 27531972

27531973 GTGCTGGGTAAATGGGTTCAAGTGTCTTTCCCTGACTTTAATGCATATGAATCAAAGG 27532032

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27532213 AGGTTGGGCTGCACTGTGGGTTTAGCAGCACCATGTGCTCAGACGGTTGAGCGCCCCAG 27532272

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27532393 CAGCGCTGCAGGCAGCCCCCTGGAGTGCTTGGGGCCTGCCCTTCACCTACAGCACACCCGA 27532452

27532453 GGCTGCTAAGTGGTGGGAGGGCATTGCCGGGCCACGCTGTGCTGCGTGAGTGCGGGCTT 27532512

27532513 GTCTGGCTCTTGAGGTACAGAGAGCGGTGGTGCCTTCCTGCAGACACCCAAGGTACAG 27532572

27532573 GGGGGGTACGCACGCGATGCTCCGACGTGGGCATCTCAGGGCACGTACCGGCTGTGTCCC 27532632

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27537193 CTTAGAATTACTTGCCTCCAGCTCCCATCTGCACAGCAAAGGGCCCTCAGTGCTCAGCCT 27537252

27537253 GGTGCTTCACGGCCTCCAGCTTTAAATGCATGGTGTCTTCTCCTGCAGACCATCAGAACTG 27537312

27537313 TGAGGGTAAACCTGTAGGGACAGCAACAGAAATGCAAGCAGTTGGGTTCAGCATGACTGA 27537372

27537373 CTGTGGATGCAGAATTATTCACAAGTGAGGAGTCGGACCACAGAACGCCGTCTTTAAGCT 27537432

27537433 GCCTGATTATTTTTTCCCTCTTTATCCTTTTTTTTTTTTTT**AG****AAGATGACATCAGGC** 27537492

27537493 **TTTTCTTGGGAACGGGTGTCAGTGTGCTCATATTATCGGTGGTTCTTGTAATTCTGATGT** 27537552

27537553 **TTAAAAATCAGCTAGAAAAAG****GT**ATCGCTTTTGTGCATTTTTTCCCTTATTTCCAAC 27537612

27537613 CACACTTTTCTTCCAGTATATTTACAATTAACACAGAAACAGTCCTTTTCTCCATGC 27537672

27537673 TCTTCTTCTCCTGAAGGCAGCTCCACCCATTAATCACATTGCTGCAGAAAAGGATATCTG 27537732

27537733 TGAATTCCACAGTATGGGAGAGAACCACTGAAAGAGCAAAAACCATTCCTCCCATGAAGC 27537792

27537793 TAAGCTTAGGGACACATGATATATTGTTATTATACTTATCTAGGGGGAATGAAAGAAGAG 27537852

27537853 ATAGGAAAAACACAACCTCTTTATGTAAGTTTTATACTAGAATTTGGTTCTGTTACTTT 27537912

Appendices

27537913 TGAATGAAGAGAGAATGCTGTCCCTGCAGGATCTGTTGGCTGCCCTCACATCTGCACC 27537972

27537973 CTGAGATTGGCAGAGGTTGGGGAGTGTACACAGCGTGACATTATCATAGAACCACAGAA 27537032

27537033 TGGCCTGGGTTGAAAAGGACCACAGTGTCAACAGTTCCAACCCCTGCTACGTGCAGGG 27538092

27538093 TCGCCAACCAGCAGCCCAGGCTGCCAGAGCCACATCCAGCCTGGCCTTGAATGCCTGCA 27538152

27538153 GGGATGGGGCATCCACAGCCTCCTTGGGCAACCTGGGAACAGTGCCTACACCCTCTGG 27538212

27538213 GTGAAAACTTCCTCCTCATATCCAACCTAAACCTCCCCTGTCTCAGTTTAAACCATTG 27538272

27538273 TCCCATCACTATTATGCTTGTCTTACTACTGTGCTGAGCAGCCTCCACAGCTGCCAC 27538332

27538333 AAATGGAACCCAGAGGAGCAGCAGAGAGAGCTGGAGAGATAGAGTTCAAATGGGAAAACC 27538392

27538392 TACCTGGACAGGGGTTTGTAACCTAATGCTTCCAAGCCCTCCACAAAAGCCATGAGTGA 27538452

27538453 TCAATACACTTAGAGTTCCTCATCATCTGGGGACAATTTTCATCCCCAGTGAACAAAGTG 27538512

27538513 AGTGACTAACAAATGGACTAACTCTTTTTTCTCTGTCTCTTTTTTTTAATGAAGATT 27538572

27538573 AAGGCAGCAGTGGCACTGGTTTTGCCAAAATGGCTGCTTGAAGATTTCCCCACATGGAA 27538632

27538633 AACAGCAAGGTGATAAAGTCCTTCCAGGTATTGCACCACTGCATCCCAGCTTTTTTTCA 27538692

27538693 CTTACATATACTTAGATTGAAAATCATTCTGCAGGTGTGAGAAATACGGACAAGAAAAGG 27538752

27538753 ATTACAGCCCTATGGGATGTAGAACTCAGAGAATCCTTTTCTCTCATTGCACTGTGCAC 27538812

27538813 AGGAGCCCAGCTTAGCTTAATCTTTAAACCTTGTCTCAATATAAATGTCATACAGTAGAA 27538872

27538873 CTGAGTCAAGCTGACCTCGAGGTACCCATGTAAGCATGGAGACATGCACAGCTCTTCTGT 27538932

27538833 CTGCTCTCCACATGTCACTGTAGTCTGCTTTGTGGAGGGGATTAGAGGAGAGAGGAGAA 27538992

27538993 AGGATGCTGAGATGAAGACCAAGAAGTCATTGACCTATTTCTCTCTTGTGATCCTCCCAT 27539052

27539053 GATATCACTCCCATTGGTGACATTTTCTGTTTCTTAGTACAGTCATCTTTTTTTCTAG 27539112

27539113 GGGAAAGAGTGAATTACAAACAGCATTTTTCAGTGAGCCGTTCTGGATAACAGTGACCCC 27539172

27539173 ACAATTACGGAAATAAAGGAGCTGTCAGGGGACAAGAAATACAAGAACGTGGACATCAAA 27539232

27539233 AAGGAACCCAGCAATGTGGTCCCTGAGAACGTGGAGCACCCACAGAGCTCAGCACCTCCC 27539292

27539293 CATAACACGGCCACGGAGGACGTCACTGACTACAAACCCAAATCTCTGATGCAAAACACA 27539352

27539353 CTAGGATATGTAGCTGCCAACATGGGCCTGATACAGCCCTACACACCAGCTCCAGAACCA 27539412

27539413 GAGACAAATATTTTCTTCCGAGACTACAGCAGCCCTTTTAGCTGCCTGTGGGATGCTGAA 27539472

27539473 GGAGCAGGGGCCAGGTGTGCTGTGACAAGATCAACCTGGTTTTAAACAACGACAGA 27539532

27539533 AGTGGACAAAACACGCATTGAGCTCCGCCAGGAGGAACAAAATGCCCTTTTGGAAAAC 27539592

27539593 CAGTGGGAAAAAACACTGTACAGTGAAGGAGTTGAGGAACAAACCTTGGTTCCAGATGAG 27539652

27539653 CTGGTTTCTTGCTTAAGGGCAATGAATGGAGGATCTGTGGATATACAGACCTGCTTTCCC 27539712

27539713 CAGAGCATCGGAAGATTGTTTGAAGTGAATGTGATGAGAAGCCACAACTTGTGACA 27539772

27539773 ATATGATGGTGACACATATCTGGAGTGAATTTGTGATGAGAAGCCACAACTTGTGACA 27539832
 27539833 ATATGATGGTGACACATATCTGCTTTTGGAGATCTCAAACATCAGAGCTGGAACAAAACCTC 27539892
 27539893 CAAATCTGAAACTCTTCCCCTTAAGTACTACAGATAGCTTTGGATGCATGGAACGCCTT 27539952
 27539953 GGCCAGTAAACATCATTTAATTTTACCTCATGTTTTTACAAACACAAAATGTGAGCTT 27540012
 27540013 AGATTCTGTAGGAGCTGAAATATAAAGCAGGTGATTCTTACAGGATGCATTCTGCTTCA 27540072
 27540073 ATTATTAGCCTAAGGCACCTCTCTTGCTAAATATACAGAACAAGTAATACCTGGAGCTGAT 27540132
 27540133 TGCCTTCAAGATGGATAAAACCATCCCCTAGGTATTCTGTCTGAATTGTGTAATAAGCT 27540192
 27540193 TGGGATCACCCATAATGAAGAGACACTACAATGAGGACAATTTACACATCACGCAGCAGC 27540252
 27540253 CATAGATACCATACGAAATCTTTTCTGTTTGACAGAATAGCCCAATTCATTTTCCTTTC 27540312
 27540313 CATTTCAGTAAGAAAAAATACATCAGATAAGAACAACCTTCATCATCTTGGTCTGCCAGC 27540372
 27540373 CCGAGTGAATATCTGTCTCCATTTGTCTACTGGGCAGATTCTTCGTTTTGGATGAAGGA 27540452
 27540453 TTTATCATCTCACATCAGTTTGTCTGCAGTTCGGCTTTGCTGCTCTGTACGAAGCAGAAC 27540512
 27540513 ATGGCAGAGAGAAATGGCATTATTTCAGTGCATAAGAGGAGATTGGATCTTCTGAGATG 27540572
 27540573 CAGAAAACAAAATATCAGAGTCTCTTTCTGCTGAGTGGTCTTGAAAGCAGTTAAAAGAT 27540632
 27540633 TGCCCAGGATGTCAGAGGTAGCCTTGCAAGATGTTCTAGAAAGCAGAAACACCCTGGGAA 27540692
 27540693 TGTGAGAGGTTGGATCATTATCACTTCTTTGACCGATCACCCCAACTTTTGGAGAAACA 27540752
 27540753 CTTGAAATGCTCTGAAGGAATACAAGCATAGAAGTTTATCATACCCTGTAAAATGGCTGA 27540812
 27540813 GCATTCCATGAGCAATGCTCTCCCTAAGGTTTGTCTACTGGTCTTCTCTCTACCTCT 27540872
 27540873 TATCACAGCAAAAACCTGTTCTTATGCCAGAGCTGCATCAGGACGTGGCAGAGCTGCATGC 27540932
 27540933 TCAGGGTGGGCAGGAGTTAGTGCCATGCAGCCAGCACTGGATGGGGCTCAGGGTGGAGCA 27540992
 27540993 AGGGGAGGGCACTGGCCCTTAAGCCTCTCCATGCTGCTCAGAGCAGGACCTTGGGCACT 27541052
 27541053 GCGGCACTATATAACAATACGTAGCACAAAGGAGCCCCTGAACTACGTACCAATACGTGT 27541112
 27541113 GATTTCCACTCACTGAGGCATCTGTGCATCTTACACAGCTCTGGGTAACGGAGCTGGCTA 27541172
 27541173 TGCACGTTGCATGGAACACACAGGGGATGTATTTGTAAAGACTGACATTAACAAAACA 27541232
 27541233 GCAAAAAAAAAAAAAAAAAAGGGAAAAAGATCACCTGACTGTGCATTACCAAAGCAAAGG 27541292
 27541293 CTGTAAACAGTCATAGCAAGACTCAGTACATTTTATAAGACTTACAAAGCTAACATAA 27541352
 27541353 ATGCTAACCTACGTGTAATTAGTTGTCTAATATTACCCACTTGATGACTGTTGGCAATAA 27541412
 27541413 AACCTGGAGATTGTAATTTTCC 27541472

Figure 7.8. Compiled genome sequence of the chIL-23R gene. Exons are in red and introns are in black. Intron splice sites are boxed. Instability motifs (ATTTA) and the polyadenylation signal (AATAAA) are underlined.